REMARKS

Claims 1–83 are pending in the present application. Claims 18–47, 53–67, 70–77 and 80 are withdrawn from further consideration as being directed to a non-elected invention. Claims 1–17, 48–54, 68, 69, 78, 79 and 81–83 of group I are under consideration. Claim 1 has been amended herein and new claims 84–89 have been added. The comments of the Examiner are addressed hereinbelow.

SEQUENCE RULES AND SPECIFICATION

Responsive to the Examiner's request to include the peptide sequences found in Figure 1, in Table 1 at pages 16 to 22, in pages 47 to 49 and in page 44 (the two (2) cited primers) in the Sequence Listing and the Notice to Comply, Applicant provides a computer readable form of the Sequence Listing as well as a paper copy thereof. The Examiner will note that the enclosed Sequence Listing includes sequence ID numbers for all the above-mentioned sequences. Responsive to the objection to the Specification, Applicant provides the attached Substitute Specification. The Substitute Specification is provided in order to comply with the requirements of 37 C.F.R. § 1.821–1.825 for applications containing nucleotide and/or amino acid disclosures and incorporates SEQ ID NOS to the nucleotide and amino acid disclosures described hereinabove.

Applicant has also taken note of the Examiner's request that the embedded hyperlink included in the description on file, namely on page 16, be deleted. In response thereto, the embedded hyperlink for BIMAS™ algorithm cited on page 16, line 3 of the description has been deleted in the Substitute Specification.

Applicant hereby states as required under 37 CFR § 1.821(h) that the content of the paper and computer readable copies of the Sequence Listing are the same and introduces no new matter. Furthermore, Applicant states that the Substitute Specification provided herewith introduces no new matter beyond the disclosure of the application as filed.

RESPONSE TO THE REJECTION OF CLAIMS 1–5, 10–17, 48–52, 68, 69, 78, 79 and 81– 83 UNDER 35 U.S.C. § 102 (b)

Applicant has taken note of the Examiner's rejection of the above-mentioned claims as being anticipated by the international application published under the no. WO 98/25962 (Jacobs K *et al.*). More specifically, the Examiner has been of the opinion that the subject matter of the above claims would already be disclosed in this international application. In other words, the Examiner has requested that the proof be provided that the claimed nucleic acid molecule is different from the one disclosed in the prior art.

Applicant also acknowledges that the Examiner has considered original claims 6 to 9 as being novel in view of WO 98/25962.

In response to this rejection, claims 2, 3, 6, and 7–10 are canceled herein for the purpose of rewriting. Claims 4, 5, and 11–83 are canceled herein without prejudice. Support for these claim amendments and new claims can be found throughout the specification and the original claims as filed. The modifications to the original claims are as follows:

claim 1 is amended herein and incorporates elements of original claim 6;
new claim 84 corresponds essentially to original claim 7;
new claim 85 is essentially a combination of original claims 1 and 8;
new claim 86 corresponds essentially to original claim 9;
new claim 87 corresponds essentially to original claim 2;
new claim 88 corresponds essentially to original claim 3; and
new claim 89 corresponds essentially to original claim10.

In view of the above-mentioned modifications, Applicant is of the opinion that the rejection against original claim 1 should be withdrawn inasmuch as the subject matter of original claims 6–9 was considered new in view of WO 98/25962.

With regard to original claims 2, 3 and 10 (new claims 87, 88 and 89), Applicant is also of the opinion that, since they ultimately depend on claim 1 or new claim 85, which

should be deemed novel in view of WO 98/25962, the rejection of original claims 2, 3 and 10 should also be withdrawn.

In response to the rejection of claims 4, 5, 11 to 17, 48 to 52, 68, 69, 78, 79, and 81 to 83, the Examiner will note that Applicant cancels the instant claims herein.

RESPONSE TO THE OBJECTION TO CLAIMS 6, 8 AND 11 (CLAIMS 1 AND 85) UNDER 37 CFR 1.75 (c)

Applicant has taken note of the Examiner's objection to original claims 6, 8 (amended claim, new claim 85) and 11 (now canceled) as improperly depending on claim 1. More specifically, the Examiner has noted that claim 1 refers to the human species while original claims 6, 8 and 11 mention Drosophila sequences. In response to the objection to original claims 6 and 8, reference to the Drosophila SEQ ID NOS:7 and 8 has been deleted in amended claim 1 and new claim 85. Therefore, only human sequences are mentioned in new claims 1 and 85. In response to the rejection of original claim 11, this claim is canceled herein.

RESPONSE TO THE OBJECTION TO ORIGINAL CLAIM 49

Applicant has taken note of the Examiner's objection to original 49 because the term "SIMP" would contain a typographical error. In response thereto, claim 49 is canceled herein.

RESPONSE TO THE OBJECTION TO ORIGINAL CLAIM 49

Applicant has taken note of the Examiner's objection to former 49 because the term "SIMP'" would contain a typographical error. In response thereto, original claim 49 has been canceled herein.

RESPONSE TO THE REJECTION OF ORIGINAL CLAIMS 14, 48, 50 AND 69 UNDER 35 USC § 112

Applicant has taken note of the Examiner's rejection of original claims 14, 48, 50 and 69 as being indefinite because of the expression "under high stringency conditions" would have unclear boundaries. In response thereto, the rejected claims have been canceled herein.

RESPONSE TO THE REJECTION OF ORIGINAL CLAIMS 1 TO 17, 48–52, 68, 69, 78 , 79 AND 81–83 UNDER 35 USC § 112

Applicant has taken note of the Examiner's rejection of the above-mentioned original claims for a lack of support for the nucleic acid molecule coding for other peptides than the three (3) peptides presented in Figure 1. More specifically, the Examiner has been of the opinion that the description on file only discloses the biological function of these three (3) peptides by mentioning that they work as T cell epitopes. The Examiner has been of the opinion that complex supplementary experiments would be necessary to adequately support the claimed invention.

With regard to the rejection of original claims 4, 5, 11 to 17, 48 to 52, 68, 69, 78, 79 and 81 to 83, since the instant claims have been canceled herein, Applicant believes that the present rejection has become obsolete.

With regard to original claims 1–3 and 7–10 (currently amended claim 1, new claims 84–89), Applicant respectfully traverses the Examiner's rejection for the following reasons.

The Examiner alleges that :

> *The specification does not establish the biological function of the newly discovered protein, other than disclosing the three peptides (as shown in Fig. 1) fragments from instant SEQ ID NO:2 encoded by SEQ ID NO:1 work as T cell epitopes.*

In response to this allegation, the Examiner is referred to the two following scientific articles published well before the filing date of the present application, namely: AL and Hughes MK *Self peptides bound by HLA class I molecules are derived from highly conserved*

regions of a set of evolutionarily conserved proteins. Immunogenetics (1995) 41:257–262; and Niedermann G *et al. Potential immunocompetence of proteolytic fragments produced by proteasomes before evolution of the vertebrate immune system.* J. Exp. Med. (1997) 185(2):209–220 (Appendices A and B, respectively).

More specifically, these two articles clearly indicate that 1) self-peptides are well-conserved among vertebrates; and 2) the mechanism by which the self-peptides are presented by an MHC molecule (for example, by proteasomes) are also highly conserved among vertebrates and throughout evolution. Consequently, Applicant submits that a person skilled in the art, with his/her general knowledge in view of these articles, would agree that the three (3) murine peptides presented in Figure 1 can scientifically be extrapolated to the human species.

Applicant has also taken note of the Examiner's argument that :

> *There is insufficient guidance regarding the parameters and sequences of peptides which correlate with the ability to be recognized by the specific CTL clone.*

In this connection, the Examiner has cited US patent no. 5,840,839 to conclude that T cell binding motifs do not necessarily equate to T cell epitopes.

Applicant traverses this argument in view of the following. Due to the high diversity of the T cell repertoire, there are no consensus sequences of epitopes that will be recognized by CTLs. Indeed, CTLs can recognize more than 10^{13} epitopes. Moreover, the Examiner is invited to note that, even though all SIMP peptides are not necessarily epitopes, all epitopes are peptides, as the ones listed in Table I of the description on file.

In this respect, the Examiner is referred to the enclosed Annex 1 (Appendix C), which shows the results of a non-published cytotoxic assay performed by Applicant on normal cells, on September 3, 2001 (i.e. before the filing date of the present application). More specifically, the Examiner will note that a CTL cell line (clone 18), that specifically recognizes peptide 18 of the MHC molecule HLA A2 on normal cells, was developed and effectively lysed control

T2 cells loaded with the synthetic peptide 18, as well as HLA A2+ cells harvested from different individuals (IND2 to IND5). Incidently, the Examiner will note that peptide 18 is included in Table I of the description on file, thus proving that enough guidance was provided regarding the sequence of the SIMP peptides which correlate with the ability to be recognized by a CTL clone.

The Examiner also alleges that:

> *The specification provides insufficient guidance with regard to these issues and provides no working examples of a peptide that would work with any MHC molecule.*

In response thereto, the Examiner is reminded that the goal of the present invention was not to provide a peptide that would work with any MHC molecule. The Examiner is referred to the Table I of the description on file, which clearly shows that the SIMP protein generates various peptides and that a high number of these peptides bind to HLA molecules. According to their amino acid sequence, the peptides will have more or less affinity with one or more molecules of the MHC complex. Therefore, the present invention contends that different peptides can bind to different HLA molecules and that a single peptide does not necessarily bind to different HLA molecules, although it could also be possible. The Examiner is reminded that the present invention resides in the provision of a nucleic acid that encodes a peptide, which in turn binds with high affinity to an HLA molecule. The invention is not limited to a peptide that would work with any MHC molecule.

The Examiner also alleges that:

> *The specification fails to teach how administration of the claimed peptide would produce a sufficient amount of CTLs, to destroy tumor cells expressing SEQ ID NO:2. Cancer therapy using immunogen is still unpredictable in the art ... In other words, only CTLs with low affinity are left, which may not be optimal for tumor elimination in vivo.*

Applicant has taken note that the documents cited by the Examiner, namely Sherman LA *et al.* (1998) and Lauritzen GF *et al.* (1998) both relate to tumor vaccination. In this connection, the Examiner is invited to note that, even though a CTL clone of low affinity can

be a limiting factor in the context of vaccination, there is no such constraint in the context of allogeneic MHC. More specifically, given the foreign nature of an allogeneic MHC complex, it will be more readily recognized by a CTL clone. Moreover, as disclosed in Luxembourg AT *et al.* (Biomagnetic isolation of antigen-specific $CD8^+$ T cells usable in immunotherapy. Nature Biotechnology vol.16: pp 281-285; published in March 1998, Appendix D), antigen-specific $CD8^+$ T cells with low affinity for the antigen can be isolated from mixed T-cell populations, specifically kill target cells *in vitro* and display an antiviral effect *in vivo*.

Furthermore, in view of the above argumentation, the Examiner is invited to note that, even T cells with low affinity for an antigen, a tumor antigen for example, can efficiently recognize their target. The Examiner is also reminded that, in order to be patentable, an invention should simply work. All the preferred embodiments included in a patent application are not necessarily efficient to the same extent. Given the disclosure of the present application, Applicant is of the opinion that a tumor antigen, in association with a SIMP peptide, as encompassed by the present invention, will effectively be recognized by a CTL clone. In this connection, the Examiner is invited to refer to the enclosed Annex 2 (Appendix E) that shows the non-published results of CTL assays performed by Applicant with the MLα018 cell line. More specifically, the Examiner will note that the MLα018 cell line can effectively lyse cells from different types of tumors (Hodgkin's lymphoma, acute lymphoblastic leukemia and Burkitt's lymphoma). Moreover, all tumor cells were HLA A2 positive. Hence, it is possible to develop CTL cell lines, in the context of the present invention, which recognize both a tumor antigen and an HLA sequence.

Finally, the Examiner is again referred to Appendix C, which also shows that an efficient T-cell clone that specifically recognizes the HLA A2 molecule on normal cells, through peptide 18 of SIMP, can indeed be developed.

The Examiner has also sustained that clonal deletion in the thymus would lead to the deletion of cells that recognize self-proteins. However, the Examiner is kindly referred to the phenomenon of autoimmunity as well as to the article by Fujita H *et al.* (1998) *Evidence that HLA class II-restricted human CD4+ T cells specific to p53 self peptides respond to p53 proteins both in wild and mutant forms* Eur. J. Immunol. 28:305–316 (Appendix F), which

both provide proof that thymic selection does not delete all the reactive clones. In support thereof, the Examiner is referred to a definition of autoimmunity: *a condition characterized by a specific humoral or cell-mediated immune response against the constituents of the body's own tissues (autoantigens); it may result in hypersensitivity reactions or, if severe, in autoimmune disease*. From the just-mentioned definition of *autoimmunity*, any person skilled in the art of the present invention would appreciate that the phenomenon of autoimmunity originates from activation of CTLs directed against self-antigens. It highlights the fact that CTL reactions against self-antigens or self-peptides such as SIMP peptides, can be envisioned.

Finally, the Examiner concludes that, in view of Lauritzen *et al.* (1998) as well as the other references discussed in the Office Action, the state of the art at the time of filing the present application was not able to predict whether or not treatment of cancer patients was possible by administering tumor antigens. The Examiner has also alleged that the specification did not provide any proof that CTLs could be generated by administration of the claimed polypeptides in order to lyse tumor cells. In response thereto, the Examiner is again referred to the article by Fujita *et al.* (1998) which clearly discloses that, although ubiquitously expressed, the p53 protein includes some peptides against which the immune system does not acquire tolerance. Accordingly, Fujita *et al.* identified several p53 self-peptide- derived epitopes against which T cells from a healthy individual responded.

Applicant has also taken note of the Examiner's rejection of original claim 78 as being drawn to a kit comprising a nucleic probe whereas the intended use for the probe would be to detect a SIMP polypeptide. In response thereto, original claim 78 has been canceled.

RESPONSE TO THE REJECTION OF ORIGINAL CLAIMS 1 TO 6, 8, 10 TO 17, 48 TO 50, 52, 68, 69, 78 , 79 AND 81 TO 83 UNDER 35 USC § 112 FIRST PARAGRAPH

Applicant has taken note of the rejection against the above-mentioned claims as failing to comply with the written description requirement. More specifically, the Examiner has been of the opinion that the rejected claims would be drawn to the genus of human nucleic acid molecules with various degrees of differences in term of structure.

First, the Examiner is invited to note that original claims 11 to 17, 48 to 50, 52, 68, 69, 78, 79 and 81 to 83 have been canceled thus rendering moot their rejection.

With regard to the rejection of original claims 1 to 6, 8 and 10, the Examiner is invited to note that mention of sequences other than human sequences have been deleted from such claims.

Furthermore, Applicant has taken note of the Examiner's rejection of original claims 1 to 3 because the specification would not establish the ubiquitous expression of SEQ ID NO:1 nucleic acid in human cells. The Examiner is invited to refer to the description on file, namely on page 23, lines 9 to 11 where it is clearly stated that SIMP sequences are ubiquitously expressed in human cells. In support thereof, the Examiner is invited to note that the expression "(not shown)" cited on page 23, line 11 of the description refers to the data presented in the enclosed Annex 3 (Appendix G), which displays tissue expression arrays performed by Applicant. These non-published arrays show a broad expression of SIMP sequences in human cells from different tissues.

The Examiner will note that original claims 4 and 5 have also been canceled herein thus rendering moot their rejection.

CONCLUSIONS

The points and concerns raised by the Examiner in the outstanding Office Action have been addressed in full, it is respectfully submitted that this application is in condition for allowance. Should the Examiner have any remaining concerns, it is respectfully requested that the Examiner contact the undersigned Attorney at (919) 854-1400 to expedite the prosecution of this application to allowance.

Applicants hereby authorize the Commissioner to charge Deposit Account No. 50-0220 in the amount of $120.00 for a one month extension of time. Applicants believe that this amount is correct. However, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 50-0220

Respectfully submitted,

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Rosa Lee Brinson

Immunogenetics (1995) 41: 257–262

HYPOTHESIS

Austin L. Hughes · Marianne K. Hughes

Self peptides bound by HLA class I molecules are derived from highly conserved regions of a set of evolutionarily conserved proteins

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Abstract An evolutionary analysis of self peptides reported to be bound by HLA class I molecules showed that these peptides are largely derived from proteins that have been highly conserved in the history of mammals. These proteins also often have universal tissue expression and have a higher than average frequency of highly hydrophilic residues. The peptides themselves are generally still more highly conserved than the source proteins and have a higher frequency of highly hydrophobic residues, evidently often being derived from conserved hydrophobic cores of the source proteins. These results suggest that the mechanism by which peptides are derived for MHC presentation may preferentially select peptides from conserved protein regions. In the case of parasite-derived peptides, such a mechanism would be adaptive in that it would reduce the likelihood of escape mutants.

Introduction

The class I major histocompatibility complex (MHC) heterodimer in vertebrates, consisting of the class I α chain and β_2-microglobulin, is assembled in the endoplasmic reticulum (ER) before transport to the cell surface. Stability of the heterodimer requires binding of a peptide in the peptide-binding groove of the class I α chain (Townsend et al. 1989). These peptides, usually nonamers, are derived from degradation of intracellular proteins mainly by means of a proteolytic complex in the cytosol, the LMP+ proteasome (Goldberg and Rock 1992; Monaco 1992; Germain 1994). Peptides are then transported across the ER membrane by the heterodimeric TAP transporter (Neefjes et al. 1993; Shepherd et al. 1993). In the case of an infected cell, a class I molecule on the cell surface binding a foreign peptide is recognized by cytotoxic T cells (CTL), resulting in the killing of the infected cell (Townsend et al. 1985; Berke 1994), whereas in non-infected cells, class I molecules bind peptides derived from self proteins (Jardetzky et al. 1991).

Understanding the process by which self peptides are selected for binding by class I MHC molecules is a key to understanding and predicting foreign peptides bound by the same molecules, but so far this process is little understood. Recently the primary structures of a number of both self and non-self peptides eluted from HLA (human MHC) class I molecules have been reported, and for many of these the source proteins have been identified by database search. To investigate the process of self peptide binding, we analyzed evolutionary conservation of these peptides and of the source proteins from which they are derived. Surprisingly, the analysis showed that HLA class I-bound self peptides are largely derived from proteins that have been highly conserved in the history of mammals and that the peptides themselves are generally even more conserved than the remainder of their source proteins.

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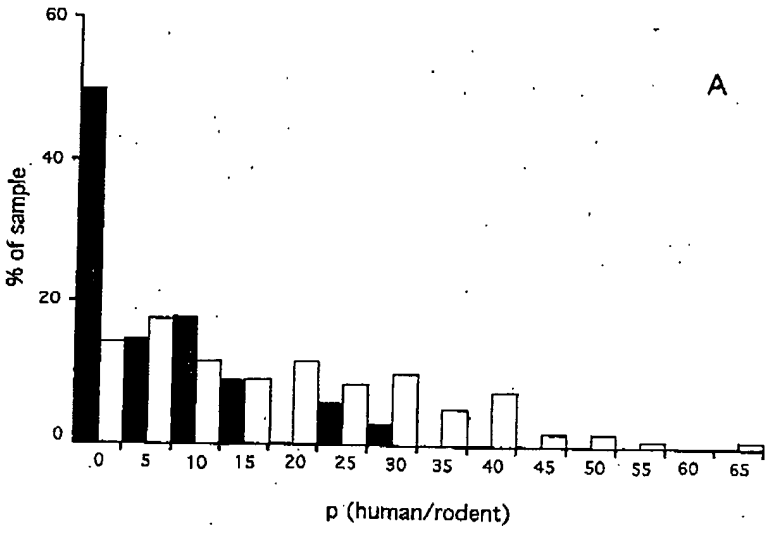
Materials and methods

Our data consisted of 34 source proteins from which self peptides have been identified (Jardetzky et al. 1991; Guo et al. 1992; Hunt and Engelhard 1992; Hunt et al. 1992; Engelhard et al. 1993; Harris et al. 1993; Huczko et al. 1993; Engelhard 1994; DiBrino et al. 1994). We chose only proteins for which an amino acid sequence was available for human and a putatively orthologous sequence was available for a murine rodent (mouse or rat). Comparison of the human and rodent orthologues, all of which diverged at the same time, made it possible to measure the relative evolutionary conservation of these proteins. The set of source proteins was compared with a random sample of 120 proteins for which putative human and murine rodent orthologues were available from the PIR database. The data set is available from the authors on request.

Although some reported self peptides are derived from HLA class I and class II molecules, these were not included in the present analysis because in most cases orthologous human and rodent loci were lacking (Hughes and Nei 1989, 1990). Peptides eluted from mouse class I



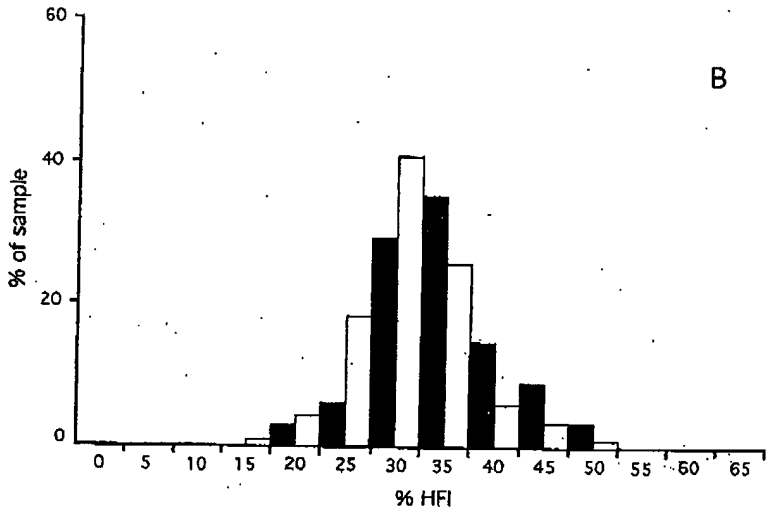


Fig. 1 A Frequency distribution (percent) of p (percent amino acid difference) between human and rodent for source proteins (*black*) and random sample (*open*); values on X axis are midpoints of frequency classes. Mann-Whitney test of the equality of median p for source proteins (2.1) and that for random sample (15.4): $P < .0001$. B Frequency distribution (percent) of %HFI (percent highly hydrophilic residues) in source proteins (*black*) and random sample (*open*); values on X axis are midpoints of frequency classes. Mann-Whitney test of the equality of median %HPI for source proteins (33.9) and that for random sample (31.1): $P < .01$

MHC molecules were also not included, so that a homologous data set was available for statistical analysis.

Results

Source proteins

In comparison with the random sample, the source proteins were found to be on average highly conserved between human and rodent. Median percent amino acid difference (p) between human and rodent was an order of magnitude lower in the source proteins than in the random sample (Fig. 1A). The same proteins also had a significantly higher median percent of highly hydrophilic residues [(D, E, K, N, Q, R) (%HFI)] than the random sample (Fig. 1B). However, there was no difference in median percent highly hydrophobic residues [(C, F, I, L, M, V, W, Y) (%HFO)] between the source proteins (median = 32.5%) and the random sample (median = 34.2%).

Table 1 Median percent amino acid difference [(human vs rodent) (p)] and percent highly hydrophilic residues (%HFI) in proteins categorized by level of expression

	Source proteins			Random sample		
Expression	N[a]	p	%HFI	N	p	%HFI
Restricted	3	14.2	25.4	48	28.6	29.4
Broad	7	7.6	32.4	43	12.4	32.2
Universal	24	1.3	35.3	29	7.1[b]	31.7[c]

[a] Occurrence of proteins in the three categories of level of expression was significantly different between the source proteins and random sample at $P < .0005$ (Chi-square test of homogeneity)
[b] Median p is significantly different between source proteins and random sample at $P < .01$ (Mann-Whitney test)
[c] Median %HFI is significantly different between source proteins and random sample at $P < .01$ (Mann-Whitney test)

Based on a survey of the literature, proteins in both sets were placed into three categories with regard to the level of tissue expression: 1) restricted, being expressed in only one cell or tissue type; 2) broad, being expressed in a number of tissues; and 3) universal, being expressed in all or nearly all cell types. There was a significant difference between the source proteins and the random sample with respect to representation of these categories, with a far higher proportion of the source proteins than of the random sample having universal expression (Table 1).

Universally expressed proteins might be expected to be highly conserved, both because many perform essential cellular functions and because a protein expressed in a wide array of tissue types may have to interact with a diverse array of other molecules. Therefore, it might be hypothesized that the source proteins are on the average more conserved than the random sample simply because universally expressed proteins account for a higher proportion of the former sample than of the latter sample. We tested this hypothesis by comparing median p in the source proteins having universal expression with that in the proteins from the random sample that have universal expression (Table 1). In this case also, median p was significantly lower in the source proteins than in the random sample (Table 1). Therefore, we can reject the hypothesis that the evolutionary conservation of the source proteins is simply a consequence of the high proportion of universally expressed proteins among the source proteins.

The universally expressed source proteins also had significantly higher median %HFI than did the universally expressed proteins from the random sample (Table 1). In fact, in the universally expressed proteins from both samples, there was a significant negative correlation between %HFI and p (Fig. 2A). By contrast, in proteins lacking universal expression, there was no correlation between %HFI and p (Fig. 2B). Therefore, the unusual evolutionary conservation of the universally expressed source proteins seems to be explainable both by their universal expression and by their high %HFI. It might be argued that the unusual evolutionary conservation of source proteins merely reflects the fact that highly conserved source proteins have been identified disproportionately, because such proteins occur disproportionately in the data base. However, even if it is true that highly conserved sequences occur disproportionately in the database, this argument is not valid because the source proteins are compared with a random sample of those in the database and are significantly more highly conserved than that sample.

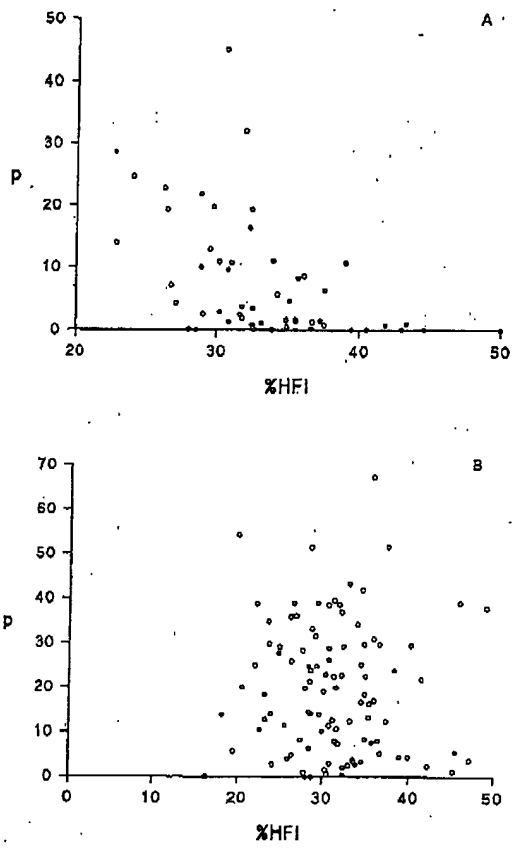


Fig. 2A, B Relationship between %HFI (percent highly hydrophilic residues) and p (percent amino acid difference between human and rodent) for A universally expressed proteins and B other proteins. *Open circles* are source proteins, *closed circles* are random sample. In A, r_s (Spearman's rank correlation coefficient) = −.585 ($P < .001$); in B, r_s = −.062 (n. s.)

Peptides

When the peptides bound by class I MHC molecules were compared with the remainder of their source proteins, the peptides were found to have significantly lower p between human and mouse than did the remainder of their source

Table 2 Comparisons between peptides bound by HLA class I molecules and the remainder of the source proteins. Abbreviations: p (% amino acid difference between human and rodent); %HFO (% highly hydrophobic residues; i.e., C, F, I, L, M, V, W, Y). Amino acid residues are numbered as in the primary translation product (precursor). A conserved stretch is a stretch of amino acid sequence showing no differences between the human and rodent sequences compared. In cases where there was no amino acid difference between human and rodent in the peptide but differences elsewhere in the source protein, the length of the conserved stretch containing the peptide (w/peptide) is compared with the length of the median conserved stretch for that protein. Wilcoxon paired-sample tests of the hypothesis that the median difference equals zero: [a]$P < .001$; [b]$P < .005$

Protein (peptide)	p		%HPO		Conserved stretch	
	Peptide	Rem.	Peptide	Rem.	W/peptide	median
C-ets-1 (154–162)	0.0	2.8	55.6	33.6	57	9.0
PCNA (241–249)	0.0	5.2	44.4	36.5	36	24.5
Cyclin D2 (189–197)	0.0	7.9	44.4	37.9	46	7.0
Calreticulin (1–10)	0.0	5.7	60.0	26.5	15	6.0
Ribosomal protein S3 (54–62)	0.0	1.3	22.2	35.9	103	63.5
Topoisomerase II (802–810)	0.0	10.8	55.6	30.8	120	3.0
Fibrillarin (173–184)	0.0	2.9	50.0	26.2	190	8.0
Prohibitin (229–241)	0.0	0.4	25.0	34.6	165	135.5
Histone H1 (49–59)	0.0	4.8	18.2	9.6	113	9.5
Histone H3 (1) (53–61)	0.0	1.7	22.2	28.5	96	18.0
(2) (73–82)	0.0	1.7	20.0	28.5	96	18.0
Elongation factor 2 (341–349)	0.0	0.8	22.2	35.6	111	103.0
Hsc70 (66–74)	0.0	0.3	44.4	29.6	427	150.0
HSP89α (201–209)	0.0	1.0	33.3	31.5	156	53.0
HSP89β (196–204)	0.0	0.7	33.3	31.9	242	107.5
Helicase p68 (1) (77–85)	0.0	1.9	22.2	29.9	22	37.0
(2) (148–156)	0.0	1.9	50.0	29.9	46	37.0
Tristetraproline (139–146)	0.0	14.2	25.0	24.8	56	4.0
C-myc (241–249)	0.0	8.4	0.0	26.7	38	4.5
Gfat (218–226)	0.0	1.0	44.4	31.5	68	52.0
Ribosomal protein L7a (26–34)	0.0	0.0	44.4	29.6	–	–
Ribosomal protein S6 (107–115)	0.0	0.0	66.7	25.4	–	–
β-actin (364–373)	0.0	0.0	33.3	35.6	–	–
Ubiquitin (63–71)	0.0	0.0	44.4	30.8	–	–
Thymosin B-10 (5–13)	0.0	0.0	22.2	17.2	–	–
Protein phosphatase-1 (113–121)	0.0	0.0	55.6	37.7	–	–
Btg1 (103–111)	0.0	0.0	55.6	33.3	–	–
Tyrosinase (371–379)	11.1	14.2	44.4	36.6	–	–
CD20 (123–131)	22.2	24.9	44.4	37.2	–	–
Cytosine methyl transferase (200–208)	22.2	23.8	44.4	28.4	–	–
Cytochrome c oxidase 2 (110–121)	33.3	28.4	50.0	45.6	–	–
Ornithine decarboxylase (259–267)	11.1	9.5	44.4	35.6	–	–
Ribosomal protein S-16 (41–49)	11.1	0.7	44.4	32.9	–	–
Iron response-element BP (848–856)	11.1	9.9	44.4	36.4	–	–
Cyclin B (313–321)	11.1	16.4	22.2	35.1	–	–
Bcl-2 (87–95)	11.1	10.6	44.4	33.0	–	–
Median difference	−1.2[a]		8.0[b]		43.8[b]	

proteins (Table 2). In fact, in 27 of 36 cases, the peptide showed no difference between human and rodent, whereas only 7 of 34 source proteins showed no difference between human and rodent (Table 2). In those cases where class I-bound peptides did not differ between human and rodent but the source protein did, we computed the length of all stretches of amino acid sequence identity between human and rodent. In 19 of these 20 cases, the stretch of sequence identity containing the peptide was longer than the median stretch of sequence identity for the protein (Table 2). Therefore, the class I-bound peptides not only are significantly more conserved than their source proteins, but they show a significant tendency to be derived from more conserved domains of these proteins.

There was a highly significant tendency for %HFO in the class I-bound peptides to exceed that in the source proteins (Table 2). Previous analysis has suggested that helper T-cell epitopes (peptides bound by class II MHC molecules) are often amphipathic in character (Margalit et al. 1987), but in the present data class I-bound peptides did not score higher on measures of amphipathicity (Auger 1993) than other peptides from the same source proteins (data not shown).

Although peptides derived from HLA molecules themselves were not included in the analysis, in this case also peptides are generally derived from relatively conserved regions. Of seven such reported peptides only one overlapped the peptide-binding region, where MHC polymorphism is concentrated; and four were derived from the relatively conserved and hydrophobic signal peptide (data not shown).

Discussion

There is experimental evidence that the mechanisms providing peptides for binding by class I MHC molecules are selective. LMP+ proteasomes cleave proteins selectively at hydrophobic and basic residues (Driscoll et al. 1993; Gaczynska et al. 1993), which constitute the majority of C-terminal residues of reported MHC class I-bound peptides; and there is evidence that the TAP transporters are selective as well (Powis et al. 1992; Neefjes et al. 1993). Such selectivity may provide a mechanistic explanation for the fact that HLA class I-bound peptides are derived from conserved, hydrophobic regions of generally hydrophilic and conserved proteins.

Both the cytosolic location of the LMP+ proteasomes and, possibly, substrate preferences of the proteases, may account for the relative hydrophilicity of source proteins; and universally expressed proteins may predominate among source proteins simply as a consequence of abundance. The negative correlation between evolutionary rate and overall hydrophilicity found in universally expressed proteins may in turn account for the fact that the source proteins are highly conserved. Both the known cleavage site preferences of LMP+ proteasomes and selectivity on the part of TAP transporters may account for the relative hydrophobicity of class I-bound peptides. Such a preference for hydrophobic peptides would also provide a bias toward conserved peptides, since hydrophobic portions of hydrophilic polypeptides tend often to be functionally important and thus conserved (e.g., signal peptides or hydrophobic cores of globular proteins). Examples in the present data include a peptide derived from a putative metal binding domain of c-myc (Vriz et al. 1989) and a peptide including a putatively catalytic tyrosine residue from topoisomerase II (Wyckoff et al. 1989).

A mechanism that biases the selection of peptides bound by class I MHC molecules toward those that are evolutionarily conserved may have adaptive aspects. Regions that are highly conserved over evolutionary time as a result of functional constraint are less likely to be polymorphic within a population than are less constrained regions (Kimura 1983). In the case of self peptides, selection of nonpolymorphic peptides eliminates selective pressure on MHC molecules to be able to accomodate a series of similar but not identical self peptides. And in the case of parasite-derived peptides, use of peptides from conserved regions as CTL epitopes reduces the likelihood of escape mutants in parasite populations.

It is known that CTL epitopes are often derived from conserved domains of parasite proteins. For example, HLA-B53, which was found to confer resistance to severe malaria in West Africa (Hill et al. 1991), binds a peptide from liver stage antigen-1 (LSA-1) of *Plasmodium falciparum* (Hill et al. 1992). Comparison of a complete sequence of one LSA-1 with a partial sequence of another allele revealed few amino acid polymorphisms in comparison with other *P. falciparum* surface proteins and none in the peptide presented by HLA-B53 (Fidock et al. 1994). Likewise, CTL epitopes of the gag protein of HIV-1 are derived from relatively nonpolymorphic regions of the protein (Johnson et al. 1991). Any mechanism that leads to preferential presentation by the MHC of such conserved regions would be advantageous to the host in combating parasitic infection.

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Potential Immunocompetence of Proteolytic Fragments Produced by Proteasomes before Evolution of the Vertebrate Immune System

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Summary

To generate peptides for presentation by major histocompatibility complex (MHC) class I molecules to T lymphocytes, the immune system of vertebrates has recruited the proteasomes, phylogenetically ancient multicatalytic high molecular weight endoproteases. We have previously shown that many of the proteolytic fragments generated by vertebrate proteasomes have structural features in common with peptides eluted from MHC class I molecules, suggesting that many MHC class I ligands are direct products of proteasomal proteolysis. Here, we report that the processing of polypeptides by proteasomes is conserved in evolution, not only among vertebrate species, but including invertebrate eukaryotes such as insects and yeast. Unexpectedly, we found that several high copy ligands of MHC class I molecules, in particular, self-ligands, are major products in digests of source polypeptides by invertebrate proteasomes. Moreover, many major dual cleavage peptides produced by invertebrate proteasomes have the length and the NH_2 and COOH termini preferred by MHC class I. Thus, the ability of proteasomes to generate potentially immunocompetent peptides evolved well before the vertebrate immune system. We demonstrate with polypeptide substrates that interferon γ induction in vivo or addition of recombinant proteasome activator 28α in vitro alters proteasomal proteolysis in such a way that the generation of peptides with the structural features of MHC class I ligands is optimized. However, these changes are quantitative and do not confer qualitatively novel characteristics to proteasomal proteolysis. The data suggest that proteasomes may have influenced the evolution of MHC class I molecules.

T lymphocytes recognize peptide fragments of protein antigens presented on the cell surface by the class I and class II molecules of the MHC. The peptide fragments are generated proteolytically inside the cell. MHC class II molecules are loaded in a secretory compartment with peptides generated in endosomes. MHC class I molecules are loaded with peptides mainly generated in the cytoplasm and transported into the ER/*cis*-Golgi by the peptide transporter associated with antigen processing (TAP[1]; 1, 2). The vast majority of peptides presented by MHC molecules are derived from self-proteins. The peptide-binding grooves of class II molecules are open at both ends and bind peptides of heterogenous length (usually 12–25 amino acids [aa]); the peptide-binding grooves of class I molecules are closed at both ends and usually bind peptides of closely defined length (8–10, mostly 9 aa). In the latter case, the peptide is usually fixed by two allele-specific anchor residues that are complementary to allele-specific pockets in the MHC class I peptide-binding groove (3, 4). In addition, H bonds are formed between relatively invariant polar aa at the ends of the binding groove and the NH_2 and COOH termini of the peptide (5). Typically, one of the allele-specific pockets, the COOH-terminal F pocket, accommodates an aliphatic, aromatic, or positively charged aa at the COOH terminus of an octa/nonamer peptide. The second anchor may

[1]*Abbreviations used in this paper:* β_2m, β_2 microglobulin; aa, amino acid; BTG1, B cell translocation gene 1; D^b, H-2D^b; hsp, heat shock protein; K^b, H-2K^b; LLnL, *N*-acetyl-1-leucinyl-1-Leucinal-1-norleucinal; MALDI-Tof-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; PA, proteasome activator; SSU, small subunit; TAP, transporter associated with antigen processing.

reside at the second, the third, or the fifth position from the NH_2 terminus of the peptide, and is more variable (3).

For the proteolytic generation of MHC class I epitopes, the immune system of vertebrates appears to have recruited the proteasomes. These ubiquitous multi-subunit endoproteases are phylogenetically ancient, as they occur in archea and bacteria, as well in eukarya. In eukaryotic cells, proteasomes appear to be the major proteolytic system of the nucleus and the cytosol. Three main forms are observed. The 20S proteasome, by itself capable of degrading misfolded or damaged polypeptides, represents the proteolytic core of the larger and more complex 26S proteasomes and the 20S–proteasome activator (PA)28 complexes (6). It has a barrel-shaped hollow structure with four layers of rings, each composed of seven subunits. The outer rings consist of proteolytically inactive α-type subunits, the inner rings of β-type subunits. Archaebacterial proteasomes possess one type of proteolytically active β subunit, i.e., all seven members in a β ring have a NH_2-terminal threonine acting as the nucleophile in peptide-bond hydrolysis. In eukaryotes, three out of seven different β-type subunits contain such a site. Proteolysis takes places inside the central cavity, between the two β rings (7, 8). In vertebrates, the three proteolytically active β-type subunits (X, Y, and Z) have IFN-γ–inducible homologues (LMP7, LMP2, and MECL1) replacing their constitutive counterparts when induced. The PA28 proteasome activator, giving rise to the 20S–PA28 complexes, is IFN-γ–inducible as well. LMP2 and LMP7, but not MECL1 and PA28, are encoded by genes in the MHC (9).

The assembly of the class I heavy chain with β_2 microglobulin (β_2m) can be substantially inhibited by peptide aldehydes, potent but not absolutely specific proteasome inhibitors (10). This seminal information, together with the discovery of the MHC-encoded proteasome subunits (11), have stimulated a host of investigations into the role of vertebrate 20S proteasomes in the processing of antigens presented by MHC class I (for review see references 6 and 9). Although most of these studies converged in suggesting a major role for proteasomes in the generation of MHC class I epitopes, it is also clear that the evidence in support of this notion remains, to some extent, circumstantial. We have recently reported that the length distribution of dual cleavage proteolytic fragments produced by mouse 20S proteasomes centers around 8–11 mer. The frequencies of individual aa at the COOH termini of proteolytic fragments generated by proteasomes correlated strikingly with that at the corresponding positions of so far eluted MHC class I ligands. For the NH_2 termini too, a significant enrichment of small and polar aa was observed for both proteasomal degradation products and MHC class I ligands (12). Intriguingly, similar COOH and NH_2 termini as well as a similar length distribution were found for peptides preferentially transported by TAP or by certain TAP alleles (13, 14). Together, these results are consistent with the notion that many of the peptides transported by TAP and many of the epitopes presented by MHC class I are directly derived by proteasomal proteolysis.

The presently available data on the substrate/ligand specificities of proteasomes, TAP, and MHC class I, in addition to suggesting coordinated function of the three systems, argue for some degree of coevolution. It has been suggested that peptide binding to MHC class I and class II may have been determined by proteolytic pathways available in ancestors before emergence of the vertebrate immune system (15). Here, we examine the hypothesis that proteasome-mediated proteolysis may have influenced the evolution of MHC class I. This hypothesis requires that the capacity of proteasomes to generate fragments with the general structural features of MHC class I binding peptides is conserved and extends back in evolution to before the emergence of MHC and T cell recognition. Although structural homologies would anticipate a high degree of conservation in proteasomal functions, differences in cleavage site usage between proteasomes at different stages of evolution have been reported (16, 17). Furthermore, it has been suggested that the IFN-γ inducible elements drastically alter the repertoire of peptide products of proteasome mediated proteolysis (18, 19). Our results suggest that the capacity of proteasomes to generate potentially immunocompetent peptides, including the efficient generation of several proven MHC class I ligands, is highly conserved in eukaryotes and evolved before the vertebrate immune system. The functional modifications by the IFN-γ–inducible elements suggest an evolutionary adaptation of proteasomes to their novel immune functions. However, these modifications appear to be mainly quantitative in nature and did not confer fundamentally novel characteristics to proteasomal proteolysis.

Materials and Methods

Reagents, Cell Lines, and Antibodies. The protease inhibitor *N*-acetyl-1-leucinyl-1-leucinal-1-norleucinal (LLnL) was purchased from Sigma Chemical Co. (St. Louis, MO); IFN-γ was from Boehringer Mannheim GmbH (Mannheim, Germany). The proteasome inhibitor lactacystin was purified as described (20). The C57BL/6-derived thymoma EL4, the human lymphoblastoid cell line T1, the human erythroblastoid cell line K562, and *Drosophila melanogaster* Schneider cells were obtained from American Type Culture Collection (Rockville, MD). Monoclonal antibodies were prepared from the hybridoma Y3 (anti-H2 class I K^b heterodimers; 21). Rabbit antiserum specific for sequences encoded by exon 8 of the K^b gene and reactive with free or β_2m-associated K^b heavy chains was a gift from Dr. S. Nathenson (Albert Einstein College, New York).

Immunoprecipitation Experiments. EL4 cells (10^7 cells/ml) were incubated for 2 h at 37°C in the presence or absence of proteasome inhibitors in cysteine and methionine-free medium, and for the last 45 min of incubation, (^{35}S) cysteine/methionine (700 μCi/ml) was added. After metabolic labeling, cells were lysed in 0.5% Nonidet P-40 (ICN Biomedicals Inc., Plainview, New York) and 0.5% Mega 9 (Sigma Chemical Co.). Samples were precleared for 60 min at 4°C with protein A–Sepharose (Pharmacia, Uppsala, Sweden) pretreated with 1 mg/ml bovine serum albumin. For immunoprecipitation, either mAb Y3 (15 μg) or 4 μl rabbit α exon 8 antiserum were added to the precleared lysates for 2 h. For the last 90 min of incubation, protein A–Sepharose was added.

Immunoprecipitates were analyzed by SDS-PAGE on 12% gels. Quantitation of gel bands was performed with the aid of a Fujix BAS 1,000 phosphorimager.

Peptides and Protein Substrates. Peptides were synthesized by using solid-phase 9-fluorenylmethoxycarbonyl chemistry in a peptide synthesizer (431A; Applied Biosystems, Foster City, CA) and subsequently purified by reverse phase HPLC. The identity of peptides was established by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-Tof-MS) and as sequence analysis on Hewlett-Packard Co. (Palo Alto, CA) instruments. Ribulose 1,5 bisphosphate carboxylase small subunit was purified from intact pea chloroplasts by denaturing continuous electrophoresis (Grimm, R., manuscript submitted).

Purification of 20S Proteasomes and of Recombinant PA28α. 20S proteasomes were purified from EL4 cells cultured with or without 50 U/ml IFN-γ for 6 d, from K562, T1, and *D. melanogaster* Schneidar cells, as well as from *Saccharomyces cerevisiae* (strain YRG-2) by fractionated precipitation of the cytosol with polyethylene glycol 6,000 followed by anion exchange chromatography on a Mono Q column (HR 5/5; Pharmacia) as previously described for EL4 cells (22). Modifications of the NaCl gradient (buffer A: 20 mM Tris/HCl [pH 7.2]; buffer B: 20 mM Tris/HCl [pH 7.2], 1 M NaCl) were as follows: *S. cerevisiae*: 0–38% B in 50 min, 38–48% B in 25 min, proteasomes eluted at 45% B; *D. melanogaster*: 0–28% B in 50 min, 28–31% B in 25 min, proteasomes eluted at 30% B; IFN-γ–treated EL4 cells: 0–34% B in 65 min, 34–37% B in 40 min, proteasomes eluted at 36% B; K562 cells: 0–37% B in 55 min, 37–41% B in 40 min, proteasomes eluted at 38% B; T1 cells: 0–35% B in 55 min, 35–38% B in 40 min, proteasomes eluted at 37% B. T1 proteasomes were further purified on a Phenylsuperose column (Pharmacia). The proteasomes were recovered in the flow-through fraction. 20S proteasomes from *Rhodococcus* sp. and recombinant *Thermoplasma acidophilum* proteasomes were purified as described (23, 24). The purity of proteasomes was assessed by SDS-PAGE followed by silver staining as described (22). Purification of recombinant human red blood cell PA28α is described in references 25 and 26.

Proteasome Digests and Analyses. Digestions of synthetic peptides (6 μg) and of the small subunit of ribulose 1,5 bisphosphate carboxylase (10 μg) with isolated proteasomes (1 μg) were performed at 37°C, except in the case of *Thermoplasma* proteasomes at 60°C, in a total volume of 300 μl buffer (20 mM Hepes/KOH [pH 7.0] 1 mM EGTA, 0.5 mM EDTA, 5 mM $MgCl_2$, 0.5 mM 2-mercaptoethanol, 0.02% sodium azide). Digestions of ovalbumin fragments Ova_{37-77} and $Ova_{239-281}$, and of the protein substrate were done in the presence of 0.004% SDS. In the experiments shown in Fig. 7, recombinant PA28α was added in a fivefold molar excess over the proteasome. Aliquots of the reaction mixture were separated by reverse phase HPLC on a SMART system equipped with a μRPC C2/C18 SC 2.1/10 column (Pharmacia). Eluent A was 0.1% (vol/vol) TFA/water; eluent B was 80% (vol/vol) acetonitril/water (0.081% TFA). The identity of peptides in individual HPLC fractions was established by MALDI-Tof-MS and as sequence analysis by Edman degradation.

For molecular mass determination without separation of the peptide products, the reaction mixtures were desalted with the help of a mini-C18 column. The peptides were then eluted by the addition of 60% acetonitril in water, concentrated, and then directly analyzed with a 62025A Maldi-Tof-MS (Hewlett-Packard Co.) using dihydroxybenzoic acid as the matrix.

For proteasome cleavage site determination, aliquots of the reaction mixtures were directly subjected to as sequence analysis by Edman degradation (pool sequencing). The sites and efficiencies of cleavage were determined from the sequence cycles and relative yields of amino acids unique in the analyzed sequence. For example, if the unique amino acid *X* appears in cycle *Y* of Edman degradation, a cleavage site is revealed *Y* residues from amino acid *X* towards the NH_2 terminus; if the unique amino acid appears in more than one cycle, several cleavage sites are revealed, their relative strengths corresponding to the relative yields of the aa in each cycle.

Results

Marked Inhibition of the Assembly of MHC Class I Molecules by the Proteasome Inhibitor Lactacystin. A functional pressure of proteasome-mediated proteolysis on the evolution of MHC class I could be envisaged if proteasomal digestion provided the major source of peptides for assembly with MHC class I. We examined the assembly of MHC class I–peptide complexes in the presence of the proteasome inhibitor lactacystin (27). Except proteasome-related particles in bacteria (28), no other protease has been reported to be inhibited by this compound. The murine thymoma cell line EL4, expressing the class I molecules H-2K^b (K^b) and H-2D^b (D^b), was incubated with or without lactacystin and metabolically labeled with ^{35}S-methionine plus cysteine. Immunoprecipitation was performed either with mAb Y3, detecting a conformational determinant of K^b present only on the assembled trimolecular complex, or with an antiserum (anti–exon 8 antiserum) that recognizes the cytoplasmic tail of K^b and D^b molecules, independent of whether a peptide is bound or not. As shown in Fig. 1, addition of lactacystin caused a marked decrease in the amount of K^b molecules precipitable with the conformation-specific antibody, but not of that precipitated by the anti–exon 8 reagent. Plateau inhibition was at ~67% as calculated by phosphorimaging (see Materials and Methods), similar or slightly better than

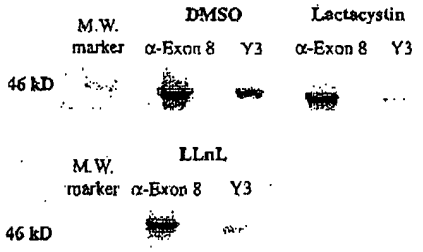


Figure 1. Lactacystin inhibits the assembly of K^b class I molecules. Autoradiograms of immunoprecipitated K^b heavy chains from ^{35}S-labeled EL4 cells. EL4 cells (10^7) were mock-treated for 2 h with DMSO, or with LLnL (100 μM), or with various concentrations of lactacystin. Concentrations of lactacystin between 100 and 1,000 μM gave maximal inhibition. Only one concentration (250 μM) is shown. [^{35}S]methionine/cysteine (0.7 mCi/ml) was added for the last 45 min of incubation. Detergent lysates were precleared and immunoprecipitated with either the conformation-dependent anti-K^b mAb Y3 or a conformation-independent anti-K^b exon 8 immunoglobulin preparation. Immunoprecipitates were separated by a 12% SDS-PAGE gel and autoradiographed.

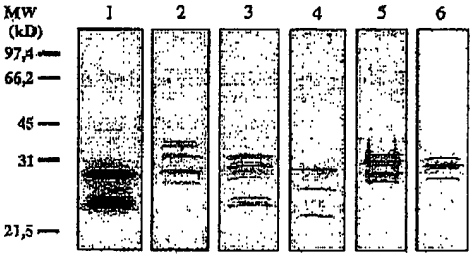


Figure 2. Polyacrylamide gel electrophoresis of purified proteasomes. Proteasomes were isolated from various organisms or cell lines as described in Materials and Methods and analyzed by SDS-PAGE on 12% gels followed by silver staining. The low molecular mass polypeptides between 22 and 35 kD represent the subunits of 20S proteasomes. Lane *1*, *Thermoplasma acidophilum*; lane *2*, *Rhodococcus sp.*; lane *3*, *S. cerevisiae*; lane *4*, *D. melanogaster*; lane *5*, mouse EL4 cells; lane *6*, human T1 cells.

that reported for the peptide aldehyde inhibitor LLnL (Fig. 1; reference 10). These data are in agreement with recent results on the inhibition of antigen presentation by lactacystin (29) and further support the notion that proteasomes participate in the generation of the majority of peptides presented by MHC class I.

Efficient Generation of Proven MHC Class I Ligands by Proteasomes of Eukaryotic Invertebrates. At early time points in the processing of short polypeptide substrates by isolated mouse 20S proteasomes, single cleavage intermediates can be detected in addition to dual cleavage oligopeptides. After consumption of the original substrate and of the single cleavage intermediates, the reaction appears to slow down and the mixture of peptide fragments approaches a relatively stable state (12). We believe that the stable state in vitro most closely resembles the conditions in vivo. Therefore, although several time points have been analyzed to compare 20S proteasomes at distinct stages of evolution (Fig. 2), we present data on the relatively stable product patterns determined after complete substrate turnover.

We have previously shown that the immunodominant ovalbumin epitope $Ova_{257-264}$ (SIINFEKL; reference 30) is the major stable product generated by mouse 20S proteasomes from the 22-mer $Ova_{Y249-269}$ as well as from the 44-

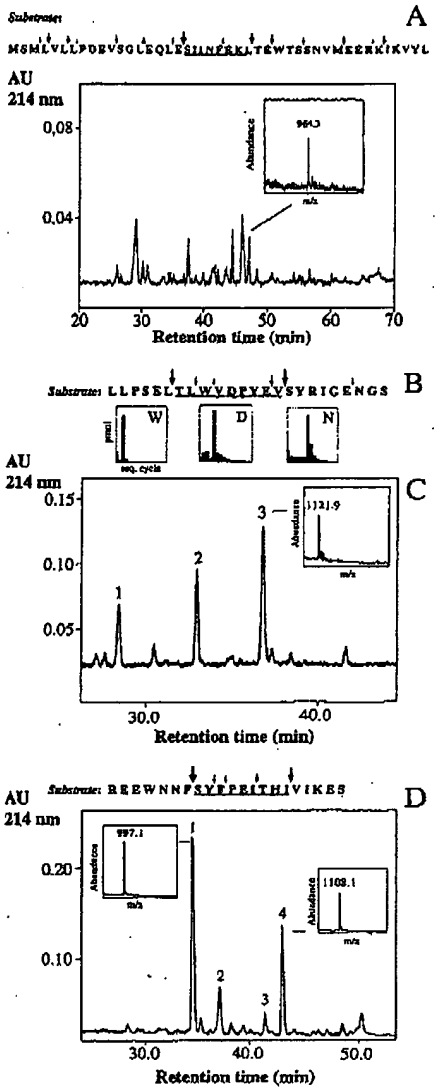


Figure 3. Proteasomes from eukaryotic invertebrates have the capacity to efficiently generate known MHC class I ligands. (*A*) Digestion of the 44-mer $Ova_{239-281}$ with 20S proteasomes isolated from *D. melanogaster* Schneider cells. The proteasome cleavage sites indicated by the arrows above the sequence were determined by Edman degradation pool sequencing (for raw data see Fig. 5 *B*). Reverse phase HPLC chromatogram of the peptide mixture is shown below. The mass spectrometry inset refers to the peak that contains the immunodominant CTL epitope SIINFEKL. (*B* and *C*) Digestion of the 24-mer $BTG1_{87-120}$ with 20S proteasomes isolated from *S. cerevisiae*. (*B*) Proteasome cleavage site determination by Edman degradation pool sequencing. For interpretation of pool-sequencing data, see legend to Fig. 5. (*C*) Reverse phase HPLC chromatogram. The numbered peaks contain the peptides LLPSEL (*1*), TLWVDPYE (*2*), and the HLA A2.1 ligand TLWVDPYEV (*3*). (*D*) Digestion of the 21-mer $JAK1_{848-868}$ with 20S proteasomes isolated from *D. melanogaster* Schneider cells. Proteasome cleavage sites indicated by the arrows above the sequence were determined from the peptide products identified in the reverse phase HPLC chromatogram shown below. The numbered peaks contain the peptides REEWNNP (*1*), REEWNNPSY (*2*), SYFPEI (*3*), and the K^k ligand SYFPEITHI (*4*). All peptide mixtures shown were analyzed after substrate consumption. The peptides contained in the peaks marked with numbers and/or mass spectrometry inserts were identified by Maldi-Tof-MS (insets) and Edman degradation (not shown).

mer $Ova_{239-281}$ (12). Here we show that this octamer is also a dominant product of digestion of $Ova_{239-281}$ by 20S proteasomes isolated from *D. melanogaster* Schneider cells (Fig. 3 *A* and Fig. 4 *A*). As a second example, we studied the generation of the nonamer TLWVDPYEV, an endogenous peptide derived from the product of the antiproliferative B cell translocation gene 1 (BTG1) and eluted as a major self-epitope from the human class I molecule HLA-A2.1 (31). Fig. 3, *B* and *C* show that this nonamer peptide is the major dual cleavage product generated by yeast (*S. cerevisiae*) proteasomes of the synthetic 24 mer encompassing this peptide in the sequence of BTG1. Moreover, we studied a 21-mer sequence derived from the tyrosine kinase JAK1 containing the nonamer SYFPEITHI, the most abundant self-peptide presented by mouse H-2K^d molecules of P815 cells (32, 33), and previously shown to be generated by digestion with mouse 20S proteasomes (34). We detected the epitope as the predominant dual cleavage product of the 21 mer with *Drosophila* proteasomes (Fig. 3 *D*). Thus, proteasomes from invertebrate eukaryotes have a high potency to generate proteolytic fragments that have been proven to serve as ligands of MHC molecules in the vertebrate immune system.

The Majority of Dual Cleavage Peptides Produced by Invertebrate Proteasomes Are in Size Range of MHC Class I Ligands. More than 90% of the peptides so far eluted from MHC class I molecules are 8–10 aa in length (3). We have recently shown that more than half of the dual cleavage proteolytic fragments generated by digestion of $Ova_{239-281}$ with mouse 20S proteasomes are 8–11 aa in length (12). In Fig. 4, *A* and *B*, we show that the peptides most efficiently produced from this substrate by *Drosophila* 20S proteasomes are in the size range of MHC class I–binding peptides (Fig. 4 *B*, *shaded area*). In addition, we studied a longer and immunologically undefined substrate, the 123-aa small subunit (SSU) of ribulose 1,5 bisphosphate carboxylase from the garden pea (*Pisum sativum L.*) (Fig. 4, *C–E*). Digests were prepared with proteasomes from mouse EL4 cells and from yeast. Most of the abundant masses in the digests represented peptide sizes of 5 to 11 aa for EL4 proteasomes or 5 to 13 aa for yeast proteasomes. In both cases, the majority of the peptides were 8–10 mer (Fig. 4, *D* and *E*, *shaded areas*). The masses of a number of abundant products are identical or nearly identical in the digests in Fig. 4, *D* and *E*, indicating that many of the peptides produced from SSU by mouse and yeast proteasomes may be identical.

Conserved General Cleavage Site Specificity of Proteasomes from Vertebrates and from Eukaryotic Invertebrates. We studied the cleavage site (...$P_3P_2P_1 - P_1'P_2'P_3'$...) preferences in polypeptides of 20S proteasomes isolated from a variety of organisms including archaebacteria, eubacteria, and nonvertebrate and vertebrate eukaryotes (see Fig. 2). Fig. 5 *A* shows the results obtained by pool sequencing of digests of the 22-mer $Ova_{Y249-269}$ containing the immunodominant $Ova_{257-264}$ (SIINFEKL) epitope. The cleavage patterns of all proteasomes of eukaryotic origin, including the murine cell line EL4, the human cell lines T1 and K562, as well as of insects and of yeast are remarkably similar; the predominant

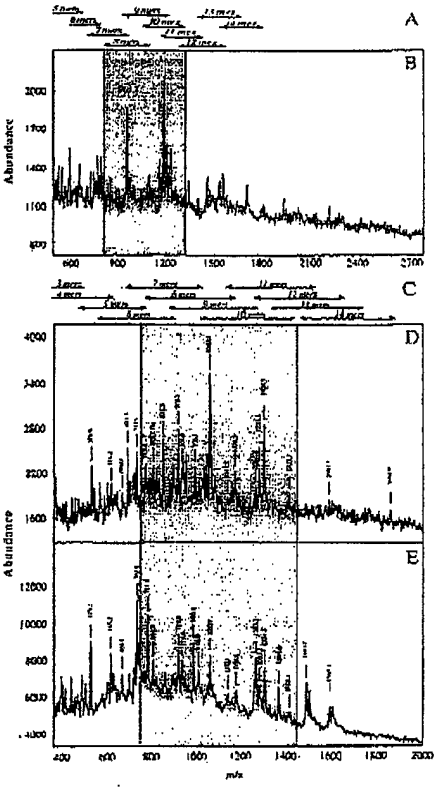
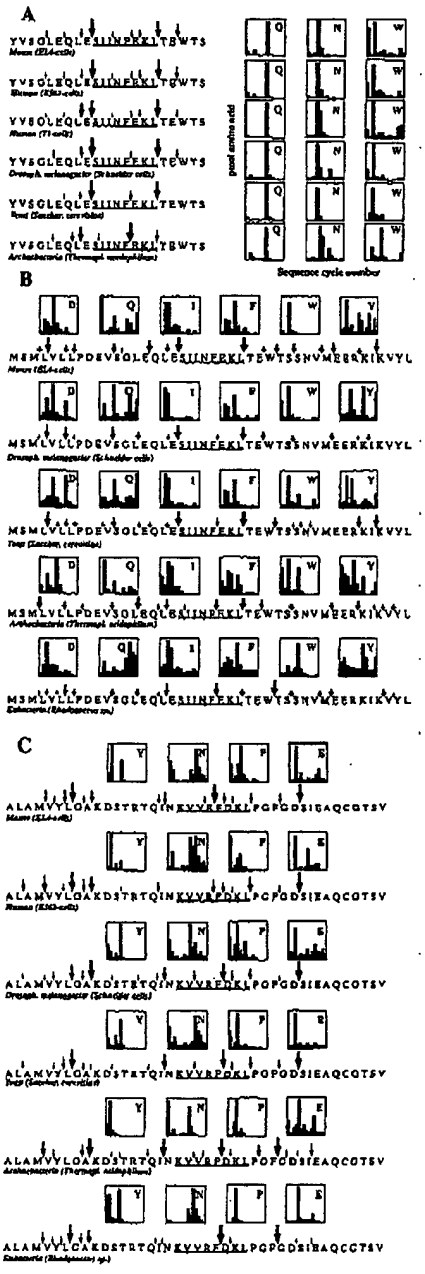


Figure 4. Most peptides efficiently produced by 20S proteasomes from invertebrate eukaryotes fall into the size range of MHC class I ligands. The 44-mer peptide $Ova_{239-281}$ (*A* and *B*) and the 123-aa SSU of ribulose 1,5 bisphosphate carboxylase (*C*, *D*, and *E*) were digested with isolated proteasomes. After substrate consumption, the resulting peptide mixtures were subjected to MALDI-Tof-MS. (*B*) Mass spectrum of the peptide mixture obtained upon digestion of $Ova_{239-281}$ with *D. melanogaster* proteasomes. The mass peak representing the immunodominant OVA epitope SIINFEKL (theoretical mass: 984.3) is indicated. (*D* and *E*) Mass spectra of the peptides mixtures obtained upon digestion of SSU with proteasomes isolated from the murine cell line EL4 (*D*) and from yeast (*E*). The shaded areas in *B*, *D* and *E* mark the mass range of 8–10-mer peptides, i.e., the typical size range of MHC class I ligands. The mass ranges of all overlapping peptides of $Ova_{239-281}$ and SSU are indicated in *A* and *C*, respectively. In the SSU digests with proteasomes isolated from EL4 cells (*D*) or from yeast (*E*), 56.7 and 58.6%, respectively, of all discernible masses fell into the size range of 8–10-mer peptides.

cleavage sites reside after the same hydrophobic (L_{264}-T_{265}) and acidic (E_{256}-S_{257}) aa. These cleavage sites precisely coincide with the NH_2- and COOH-terminal epitope boundaries. In contrast, the cleavage pattern of archaebacterial proteasomes is clearly different; they prefer to cleave after



aromatic (F_{251}-E_{262}) and aliphatic aa (L_{254}-T_{265}, L_{255}-E_{258}), no cleavage after acidic aa is seen, and the major cleavage site destroys the epitope. Analyses of the degradation of longer substrates is shown in Fig. 5, *B* and *C*. The 44-mer $Ova_{239-281}$ (Fig. 5 *B*) represents a longer fragment containing the immunodominant SIINFEKL; the 41-mer Ova_{37-77} (Fig. 5 *C*) contains the poorly immunogenic epitope Ova_{55-62} (KVVRFDKL), also presented by K^b (22, 35). For bacterial proteasomes, the data in Fig. 5 *C* highlight the preference for aromatic and aliphatic aa in P_1, whereas cleavage after charged aa is rare but not impossible (Fig. 5 *B*). Cleavage patterns of all eukaryotic examples, although not fully identical, reflect the same broad but characteristic P_1 specificity spectrum. About 60–65% of the peptide bonds hydrolyzed (e.g., 11/17 in $Ova_{239-281}$ by mouse EL4-proteasomes; 11/18 by yeast proteasomes) have an aromatic or a hydrophobic aliphatic aa in the P_1 position. Most of the remaining peptide bonds have either a positively (R) or negatively charged (E, D) aa in the P_1 position. In addition, together with results in Fig. 3 (see above), these data extend to invertebrate eukaryotes our previous finding that proteasomes have a preference for small or polar aa in the P_1' position of the scissile bond (12). Major cleavage sites are: E_{256}-S_{257} and L_{264}-T_{265} in $Ova_{Y249-289}$ (Fig. 5 *A*), L_{102}-T_{103} and V_{111}-S_{112} in $BTG1_{97-120}$ (see Fig. 3, *B* and *C*), and F_{354}-S_{355} in $JAK1_{348-358}$ (see Fig. 3 *D*).

Functional Effects of IFN-γ–Inducible Elements of Vertebrate Proteasomes. The existence of IFN-γ–inducible proteasomal elements in vertebrates indicates that the proteasomes themselves have evolved by adapting to their novel immunological role. If the specificity of proteasomal proteolysis was drastically and qualitatively altered by the IFN-γ–inducible elements, a significant restricting role of proteasomes in the evolution of MHC would be less likely. Most previous studies on the functional effects of the IFN-γ–inducible β subunits LMP2, LMP7, and MECL1 used short (3–4 aa) fluorogenic substrates, and inconsistent changes in peptidase activities have been reported by different investigators. By digesting polypeptide sequences more likely to resemble physiological proteasome substrates, we compared proteasomes from untreated with that of IFN-γ–treated EL4

Figure 5. Proteasomes from vertebrates and from eukaryotic invertebrates show highly conserved cleavage patterns in polypeptides. Synthetic peptides $Ova_{Y249-289}$ (*A*), $Ova_{239-281}$ (*B*), and Ova_{37-77} (*C*) were incubated in the presence of 20S proteasomes isolated from the indicated cell lines or organisms. After substrate consumption, the mixtures were subjected to pool sequencing by Edman degradation. Proteasome cleavage sites were determined and quantitatively estimated by the sequence cycle numbers and the yields of unique amino acids. For example, the strong signal for asparagine (*N*) in sequence cycle 4 (panel *A*) indicates a strong cleavage site four residues towards the NH_2 terminus of N between *E-S*. Although isoleucine (*I*) is not unique, these signals are necessary for the interpretation of the phenylalanine (*F*) signal, and are therefore included in panel *B*. I undergoes racemization to iso- and alloisoleucine, the latter representing 30-40% and coeluting with F. The phenylalanine signals in cycles 2 and 3 are therefore caused by isoleucine (e.g., EL4 digest). Cleavage sites are indicated by arrows, with the sizes reflecting estimates of the relative efficiency of cleavage. The CTL epitopes SIINFEKL and KVVRFDKL are underlined.

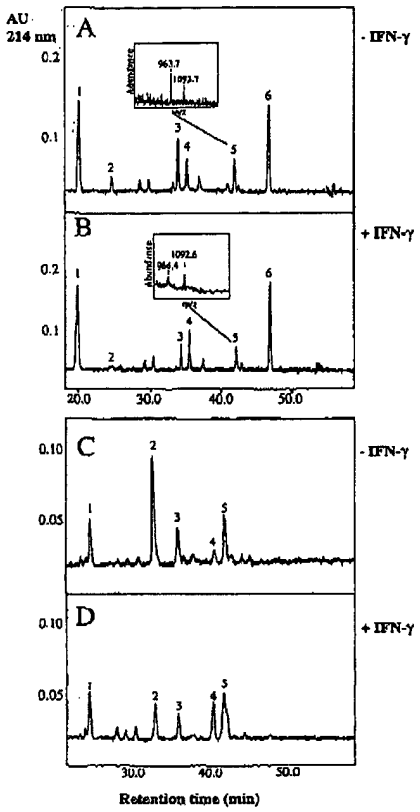


Figure 6. Proteasomes from IFN-γ–treated cells generate more peptides with hydrophobic and fewer peptides with acidic COOH termini from polypeptides. The substrates $Ova_{Y249-269}$ (*A* and *B*) and $BTG1_{97-120}$ (*C* and *D*) were incubated in the presence of 20S proteasomes from untreated EL4 cells (*A* and *C*) or EL4 cells treated with IFN-γ (*B* and *D*). The sequences of the substrates are given in Fig. 5, *A* and *B*, respectively. After consumption of the substrates, the peptide mixtures were separated by reverse phase HPLC. Peptides contained in the peaks marked with numbers are: (*A* and *B*) TEWTS (*1*), YVSGLE (*2*), YVSGLEQLE (*3*), YVSGLEQL (*4*), ESIINFEKL and the K^b ligand SIINFEKL (*5*), and SIINFEKLTEWTS/ESIINFEKLTEWTS (*6*); (*C* and *D*) LLPSEL (*1*), TLWVDPYE (*2*), TLWVDPYEVS (*3*), the HLA-A2.1 ligand TLWVDPYEV (*4*), and TLWVDPYEVSY (*5*). Peptides were identified by MALDI-Tof-MS and Edman degradation.

cells. Enhanced expression of LMP2 and LMP7 in induced compared to uninduced cells was monitored by Western blot analyses (not shown). Fig. 6, *A* and *B* show the HPLC patterns obtained upon digestion of $Ova_{Y249-269}$. Fig. 6, *C* and *D* that of the BTG1-derived 24 mer. In both cases, we observe that proteasomes from IFN-γ–treated cells generate increased amounts of fragments with hydrophobic COOH termini (YVSGLEQL is peak *4* in Fig. 6, *A* and *B*, and TLWVDPYEV is peak *4* in Fig. 6, *C* and *D*) and decreased amounts of fragments with acidic COOH termini (YVSGLE is peak *2*, and YVSGLEQLE is peak *3* in Fig. 6, *A* and *B*; TLWVDPYE is peak *2* in Fig. 6, *C* and *D*). This is in line with general preferences of MHC class I molecules, although it does not necessarily result in improved production of each individual epitope; production of $BTG1_{103-111}$ (TLWVYPDEV, peak *4* in Fig. 6, *C* and *D*) is improved, whereas production of $Ova_{257-264}$ (SIINFEKL, contained in peak *5*, theoretical mass: 963.14, in Fig. 6, *A* and *B*) is impaired. In spite of these quantitative changes, however, the same set of major proteolytic fragments is produced by proteasomes isolated from uninduced and from IFN-γ–induced cells.

The IFN-γ–inducible PA28 enhancer, existing in two homologous forms, α and β (25, 36), binds as a ring-like hexa/heptameric structure to the α endplates of the 20S proteasome (37). PA28-capped 20S proteasomes exhibit enhanced activity towards short fluorogenic model substrates (26, 38–40). Here we analyze the effects of recombinant PA28α on the digestion of the 22-mer $Ova_{Y249-269}$ by 20S proteasomes (Fig. 7). Addition of PA28α leads to a slightly increased turnover of the substrate and reverses the ratio between HPLC peak *4* (SIINFEKL and ESIINFEKL) and HPLC peak *5* (SIINFEKLTEWTS and ESIINFEKLTEWTS). The peptides in peak *4* represent dual cleavage fragments; those in peak *5* are produced by a single cleavage only. Thus, in the presence of PA28α, the rate of accumulation of dual cleavage peptides is relatively increased, in line with results recently reported for native PA28 presumably consisting of both α and β isoforms (34). However, the dual cleavage peptides that are more efficiently produced in the presence of the activator are generated also in its absence. Together, these results support the notion that the IFN-γ–inducible proteasomal elements, including PA28 as well as the inducible β-type subunits, modify the 20S-proteasome in such a way that the generation of immunocompetent peptides is quantitatively improved, without drastic alterations of the specificity of proteasomal proteolysis.

Discussion

The work presented in this paper was stimulated by our observation that each of three proven MHC class I ligands was found as a major proteolytic fragment upon digestion of precursor polypeptides with yeast and/or insect proteasomes. One possible way to rationalize this observation was that the preexisting proteolytic fragments of proteasomes provided an important evolutionary force in shaping the peptide binding groove of MHC class I molecules. Such a mechanism would predict that proteasomal digestion would be the major source of MHC class I ligands in immunologically competent vertebrates. The evidence in favor of this notion is in part circumstantial and is a matter of continu-

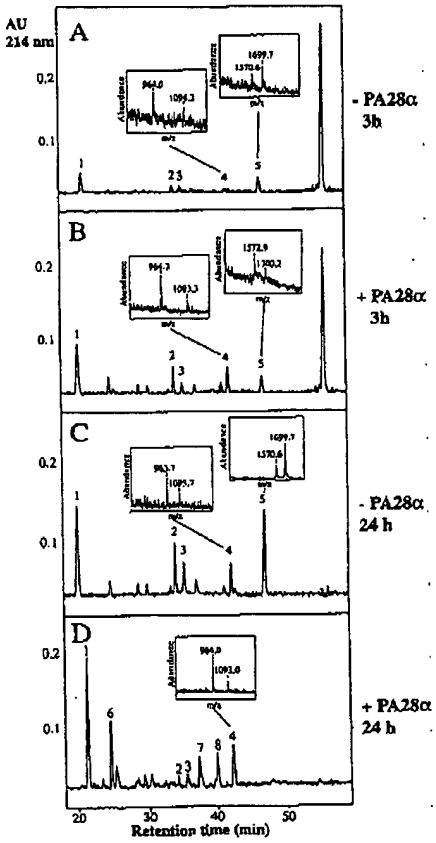


Figure 7. PA28α enhances the rate of accumulation of dual cleavage products without changing the cleavage site specificity of the proteasome. The substrate $Ova_{Y249-269}$ (for the sequence, see Fig. 5 *A*) was incubated with 20S proteasomes from EL4 cells in the absence (*A* and *C*) or presence (*B* and *D*) of recombinant PA28α. At the time points indicated, the mixtures were separated by reverse phase HPLC. The peptides in the peaks marked with numbers are: TEWTS (*1*), YVSGLEQLE (*2*), YVSGLEQL (*3*), ESIINFEKL and the K^b ligand SIINFEKL (*4*), SIINFEKLTEWTS and ESIINFEKLTEWTS (*5*), YVSGLE (*6*), SIINFE (*7*), and SIINF (*8*). The large peak at the right of *A* and *B* is undigested substrate. Peptides were identified by MALDI-Tof-MS and Edman degradation.

ing debate. We show that lactacystin, specific proteasome inhibitor until otherwise demonstrated, inhibits the assembly of about two-thirds of newly synthesized MHC class I heavy chains with β_2m. Inhibition of recognition of a panel of cytotoxic T lymphocyte epitopes by lactacystin has recently been reported (29). Although protease inhibitors cannot provide definitive proof, the combined data on this and other (10) proteasome inhibitors suggest that proteasomes are critically involved in the generation of peptides for assembly with MHC class I. In addition, data from this and other laboratories (12, 34, 41) suggest that proteasomes often are involved in the final proteolytic steps of epitope generation.

A given MHC class I molecule can accommodate large arrays of different peptides. Typically, peptide specificity is constrained by two binding pockets, whereas the rest of the peptide sequence may vary. This is reminiscent of many endoproteases whose active sites can accommodate many different peptide sequences. In many of these enzymes, the primary determinant of substrate specificity is the S_1 subsite, accommodating the P_1 residue of the scissile bond P_1–$P_{1'}$. The F pocket of MHC class I molecules accommodates the side chain of the aa at the COOH terminus of the peptide with a strong preference for aliphatic, aromatic, and charged aa. Except for negatively charged residues, the P_1 preference of proteasomes closely resembles the preferences of the F pocket. The F pocket may therefore represent the structural center of the relationship of MHC class I molecules to the proteasome. Nevertheless, no class I allele has been found so far whose F pocket prefers peptides with acidic COOH termini, although these are also efficiently produced by proteasomes of vertebrates and invertebrates. This may be taken as an indication that MHC class I molecules evolved independently of proteasomes and that their preference for aliphatic, aromatic, or positively charged COOH termini has other reasons. However, it is also possible that there was selection against acidic amino acid side chains in the COOH-terminal ligand position. For example, it is conceivable that an acidic COOH-terminal aa side chain interferes with the formation of the H-bond system between conserved polar residues of class I and the free backbone carboxylate of the ligand. During the approach of a peptide with an acidic COOH-terminal aa side chain, the side-chain carboxylate, instead of the backbone carboxylate, may become engaged in H-bond formation, preventing proper anchoring of the ligand in the pockets of the peptide binding groove. Furthermore, peptides with acidic COOH termini may not be correctly handled by other components of the processing and presentation machinery, such as heat shock proteins (hsp), which have been proposed to shuttle peptides in the class I pathway (42), or TAP. However, at least human TAP has been shown to translocate peptides with acidic COOH termini (43).

Our pool-sequencing data strongly support the high degree of conservation among eukaryotes of the cleavage site usage in polypeptides by 20S proteasomes. Although cleavage efficiencies of individual peptide bonds are not always identical, the P_1 specificities of both vertebrate and invertebrate eukaryotic proteasomes are confined by the same broad, but well-defined, limits. The same applies to the $P_{1'}$ position where we frequently see small or polar side chains, particularly in major cleavage sites. For example, in the BTG1-derived sequence, yeast 20S proteasomes most efficiently hydrolyzed bonds that contain a small and/or polar $P_{1'}$ residue (L-T, V-S), in addition to a suitable P_1 residue. Similar preferences were seen in $Ova_{Y249-269}$ (E-S, L-T) and

$JAK1_{348-368}$ (F-S). Statistical analysis of so far eluted MHC class I ligands revealed an enrichment of small and polar residues in the NH_2-terminal position (most significantly S, but also G and A) (12). In addition, a recent analysis of D^b-binding ligands has shown a significant enrichment for S, T, and C in the NH_2-terminal position of high affinity peptides (44). Moreover, small polar peptide NH_2 termini favor hydrogen bond formation in HLA-B27 (5). Also, TAP is known to have enhanced binding/translocation efficiency for peptides with small or polar residues (45; P. van Endert, personal communication). We favor the hypothesis that these structural features of the peptide-binding groove of MHC class I, and perhaps also of the peptide-binding site of TAP, may represent evolutionary adaptations to conserved features of proteasomal proteolysis.

A striking property of proteasomes is the defined length of their proteolytic fragments. For archaebacterial proteasomes, peptide length centers around 7 and 8 mers (46). Upon digestion of 22- and 44-mer OVA-derived polypeptides by mouse 20S proteasomes, we have recently observed that the majority of the dual cleavage peptide products were 8–11 mer. Peptides of this length dominated among the dual cleavage products at all time points tested, including early in the time course. Many of the peptides of this length are relatively stable, even upon prolonged digestion by proteasomes (12). Most MHC class I molecules bind 8–10/11-mer peptides (3). Nonamers seem to be preferred by most alleles. In this report, we show that a major proportion of the stable peptides generated by yeast and *D. melanogaster* proteasomes fall into the size range preferred by MHC class I. Thus, the putative evolutionary relationships between proteasomes and MHC class I may include the length of the peptide-binding groove. In addition, the highly conserved clusters of polar aa at both ends of the peptide binding groove may have evolved to facilitate efficient H-bond formation with the ends of the short peptides produced by proteasomes.

IFN-γ is a pivotal cytokine in the function of the immune system and the incorporation of IFN-γ–inducible elements into the structure of proteasomes is highly suggestive of an evolutionary adaptation to the requirements of the immune system. One of the IFN-γ–inducible β subunits, LMP7, is first detected in the nurseshark (47), i.e., at the same phylogenetic step as most other elements of the vertebrate immune system (48). It was therefore possible that MHC molecules evolved independently of proteasomes followed by a unidirectional adaptation of proteasomes to the requirements of MHC class I. On the other hand, as shown in the present paper, the ability of proteasomes to generate potentially immunocompetent peptides preceded the evolution of the MHC, suggesting the reverse order of adaptation. We were therefore interested in understanding the extent of functional modification inflicted upon proteasomes by IFN-γ–inducible elements.

Based on experiments with fluorogenic tri- and tetrapeptides, IFN-γ–dependent alterations in the substrate specificity of proteasomes were reported. In the first reports on this subject, the authors observed that proteasomes isolated from IFN-γ–treated cells gained about twofold higher chymotrypsin-like (Suc-LLVY-MCA–hydrolyzing) and trypsin-like (Boc-LLR-MCA–hydrolyzing) activities (49, 50), but lost about half of the postglutamyl (Cl_2-LLE-MNA–hydrolyzing) activity (49). The authors suggested that these functional alterations should favor the degradation of proteins to peptides that terminate in hydrophobic and basic residues that are usually found bound to MHC class I. These results were confirmed by some (51), but not by others (18, 52, 53). Studies using polypeptide substrates have yielded results inconsistent with each other and with that obtained with fluorogenic substrates (18, 41). Taken together, the functional consequences of the incorporation of IFN-γ–inducible β subunits remain incompletely understood.

Using as substrates 22- and 24-mer polypeptide sequences corresponding to natural proteins, we show that the characteristic P_1 specificity spectrum of proteasomes remains qualitatively unchanged in proteasomes isolated from IFN-γ–treated cells. However, as shown for the first time with polypeptide substrates, we see enhanced hydrolysis of individual peptide bonds with hydrophobic P_1 residues by proteasomes of IFN-γ–treated cells compared to that of untreated cells. In addition, we see reduced cleavage of neighboring peptide bonds with acidic P_1 residues. Thus, the data presented here for polypeptide substrates agree with that first reported by Gaczynska et al. with short fluorogenic substrates (49). However, together with our results, the rather mild defects of mice genetically deficient in LMP2 or LMP7 (54, 55), as well as the restoration of antigen presentation in LMP2/LMP7/TAP triple-deficient T2 cells by transfection with TAP alone (56–59), argue against drastic qualitative alterations in the cleavage preferences of proteasomes by IFN-γ–inducible β subunits.

Another IFN-γ–inducible element is the enhancer PA28. Recently, Dick et al. reported for the degradation of 19–25-mer substrates by 20S proteasomes, a substantially enhanced rate of accumulation of dual cleavage products by addition of PA28 (34). Here we show that recombinant PA28α is sufficient to induce this effect. This is in line with the recent finding that transfection of PA28α is sufficient to improve the recognition of virus-infected cells by CTLs (60). However, with and without PA28, the same cleavage sites are used and the same products are generated. Thus, the highly conserved general cleavage specificity of the 20S proteasome remains unchanged in the presence of PA28. Nevertheless, PA28 might have been evolved to optimize the capacity of the 20S proteasome for oligopeptide generation, in particular from short substrates.

Evolutionary relationships have been invoked between MHC class I and the hsp70 family of chaperones (61, 62). However, recent structural studies (63, 64) indicate that the homologies between the peptide-binding regions of hsp70 and MHC molecules are less than originally anticipated. Observations suggesting evolutionary links between proteasomes, MHC, TAP, and perhaps hsp70 were recently reported by Kasahara et al. (65) and Katsanis et al. (66). Both the human and the mouse genomes contain three regions with striking homology to the MHC complex, as they com-

prise genes coding for proteasome β-type subunits, ABC transporters, hsp70, NOTCH, and complement components. One of these regions, in addition, harbors the gene for CD1. They speculate that the MHC complex and these homologous regions might have been generated by duplication of an ancestral syntenic group in jawless fish, i.e., before emergence of T cell recognition. This implies that MHC class I–like molecules, and perhaps also TAP, may have existed before the adaptive immune system.

Due to their role in the degradation of unfolded polypeptides, proteasomes are adapted to cleave hydrophobic sequences from the inside of proteins. Most of the abundant self-peptides eluted from MHC class I are derived from highly conserved hydrophobic regions of a restricted set of evolutionary conserved ubiquitous intracellular proteins (67). In view of the possible existence of MHC class I before T cell recognition, it is conceivable that the functional cooperation between proteasomes and the precursor of MHC class I was originally designed to present self-peptides and to serve a purpose other than self-nonself discrimination, for example, inhibition of NK killing. MHC polymorphism and T cell recognition may have evolved subsequently, thus accommodating the greater variety of foreign peptides.

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Note added in proof. While this article was in print, the x-ray structure of the yeast 20S proteasome was reported (Groll, M., L. Ditzel, J. Löwe, D. Stock, M. Botchler, H.D. Bartunik, and R. Huber. 1997. Structure of the 20S proteasome from yeast at 2.4 A resolution. *Nature (Lond.).* 386:463–471). The results are in excellent agreement with the length distribution of proteasomal fragments and with the alterations by IFN-γ–inducible elements described in this paper.

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ANNEX 1

03-09-01 Cytotoxicity assay on normal cells

	CTL anti-18		
Targets	Effector :target ratio	1.25 :1	0.6 :1
Negative control	T2 cells + irrelevant peptide	37%	20%
Positive control	T2 cells + peptide18	63%	46%
HLA A2+	IND1	0%	0%
	IND2	52%	34%
	IND3	59%	49%
	IND4	63%	44%
	IND5	56%	37%
HLA A2-	IND6	0%	0%
	IND7	0%	0%
	IND8	0%	0%
	IND9	0%	0%
	IND10	40%	30%

Peptide 18 is presented by HLA A2

Biomagnetic isolation of antigen-specific CD8⁺ T cells usable in immunotherapy

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Isolating antigen-specific T lymphocytes is hampered by the low frequency of the cells and the low affinity between T-cell receptors (TCR) and antigen. We describe the isolation and purification of antigen-specific CD8⁺ T lymphocytes from mixed T-cell populations. Magnetic beads coated with major histocompatibility complex class I molecules loaded with specific peptide were used as a substrate for T-cell capture. Low-frequency T cells, as well as T cells with TCR of low affinity for the antigen were captured on the beads. Following isolation and expansion, recovered cells specifically killed target cells in vitro, and displayed antiviral effect in vivo.

Keywords: applied immunology, cell purification

Antigen-specific immune responses are mediated by antigen-specific effector B and T lymphocytes. These cells originate from resting precursor cells expressing receptors for various antigens that, upon encounter with specific antigens and appropriate costimulation, become activated, expand, and differentiate into effector cells. Adoptive T-cell therapy represents a promising approach to treat cancer and combat persistent viral infections[1]. Its development is limited by the low frequency of antigen-specific precursor lymphocytes. For instance, virus-specific cytotoxic T-lymphocyte (CTL) precursor (CTLp) frequencies in the peripheral lymphoid tissues of mice are generally <1/100,000–1/1,000,000 (ref. 2,3). Isolation of antigen-specific lymphocytes has been described in the case of mouse spleen resting B cells specific for trinitrophenyl (TNP)[4]. Using a rosetting step on haptenated horse red blood cells allowed the recovery of hapten-specific B cells with a 40% purity. This was a very favorable situation because of the relatively high frequency of B cells specific for TNP (about 1%)[5]. Low precursor frequency is a problem with T cells. In addition, while B cells recognize antigen directly, T cells recognize a complex structure consisting of an antigenic peptide bound to a major histocompatibility (MHC) molecule. T-cell receptor (TCR)/MHC-peptide interaction has a low to moderate affinity (10^4–10^7 M^{-1} range)[6–8]. Antibodies usually exhibit affinities several orders of magnitude higher and exploit multivalency. New techniques for isolation of rare cell populations, based on cell sorting and/or magnetic separation, are available[9–11]. Also, recombinant ligands for TCR are available, produced by combining recombinant empty MHC molecules[12] and MHC-binding antigenic peptides[13–15]. These synthetic MHC-peptide complexes can be immobilized on beads to yield multivalent ligands for the TCR. Theoretically, multivalency should help to overcome low affinity. The interaction between TCR and immobilized peptide-MHC complex has been shown to lead to the establishment of stable interactions in certain in vitro systems[16,17]. In two of these reports, TCR/MHC-peptide interactions were not the primary mediator of adhesion; rather, they initiated signal transduction events that led to activation of adhesion via accessory molecules.

We describe a method to isolate antigen-specific T cells based on an artificial substrate for T cells displaying a high density of identical MHC-peptide complexes. This substrate consists of empty MHC molecules purified from *Drosophila melanogaster* cells[18] immobilized on magnetic beads and loaded with peptide. We tested the ability of this substrate to specifically capture T cells in an antigen-dependent manner using T cells purified from mice transgenic for the 2C TCR[19]. This system is used to purify antigen-specific T cells starting from mixed lymphocyte populations.

Results and discussion

Capture of 2C T cells on MHC-coated magnetic beads. The 2C TCR recognizes several peptides in association with L^d (refs. 8,9), as well as with K^{bm8} and K^b (refs. 19–21) with affinities in the 10^4–10^7 M^{-1} range (Tables 1 and 2). Upon incubation at room temperature in the presence of high-affinity peptide QL9, intermediate-affinity peptide p2Ca, or low-affinity peptide SL9, the majority of 2C T cells attached to L^d-coated beads, as judged by microscopic examination after 4 hours of incubation (Table 1). No cell attachment occurred in the presence of non-2C reactive L^d-lymphocytic choriomeningitis virus (LCMV) and L^d-murine cytomegalovirus (MCMV) peptide complexes. Flow cytometry was used to quantitate the formation of complexes. 2C T cells were labeled with fluorescein, and beads were labeled with phycoerythrin. Analysis of green versus red fluorescence dot plots (Fig. 1) showed that in the presence of QL9, p2Ca and SL9 cell-bead complex formation was clearly observed with over 85% of the cells shifting to a high red fluorescence value. Some red-colored events (2.2%) were detected in the sample incubated with a control peptide (LCMV); this was most likely due to nonspecific adsorption of a small amount of fluorescein-labeled cell debris to the beads, as no attached cells were seen by microscopic examination under these conditions. Forward versus side scatter measurements were used to confirm the above results, because cells and beads had very different forward and side scatters (Fig. 2). Eighty-one percent and seventy-two percent of the cells were adsorbed to L^d-coated beads in the presence of QL9 and p2Ca, respectively. Several populations, which differed by their side scat-

ter values, were visible in the complex region; they likely represented complexes containing different numbers of beads. In these experiments, we used saturating amounts of MHC molecules to coat beads in order to achieve maximal T-cell capture. With a threefold lower density of L^d, for instance, cell adsorption was unchanged in the presence of high-affinity peptide QL9, but significantly (approximately 60%) decreased with intermediate-affinity peptide p2Ca, and undetectable with low-affinity peptide SL9 (Fig. 3). This indicates that altering MHC density is a way to select specific subsets of T cells. We also examined K^{bm3}- and K^b-peptide complexes for cell adsorption. These complexes are recognized by the 2C TCR with affinities between 10^6 and 10^8 M^{-1}, in the range of typical syngeneic TCR/MHC-peptide interaction. In the presence of 2C reactive K^{bm3}-dEV-8, K^{bm3}-SIYR, and K^b-SIYR complexes, 59.2%, 73.9%, and 62.6% of the cells were adsorbed to beads, respectively. In the presence of non-2C reactive L^d-LCMV, K^{bm3}-E1, and K^b-E1 complexes, 2.6%, 1.1%, and 0.2% of events, respectively, were found in the complex region. Capture was not completely correlated with TCR-ligand affinity, as we consistently observed capture with K^{bm3}-dEV-8 (1.8×10^6 M^{-1}), K^b-SIYR (3.1×10^7 M^{-1}), or K^{bm3}-SIYR (3.4×10^7 M^{-1}), but not with L^d-p2Ca-A3 (2×10^7 M^{-1}) or K^b-dEV-8 (1.2×10^6 M^{-1}) (affinities from refs. 8 and 9 and data not shown). Attachment of cells to beads was time dependent, with remarkably parallel kinetics of adsorption for various peptide-MHC complexes. Binding was detectable after 5 minutes of incubation, increased to reach a plateau between 1 and 4 hours, and then decreased, perhaps due to the initiation of a deadhesion process[11]. Attachment was comparable at room temperature and at 37°C, with the exception of L^d-p2Ca, for which attachment levels at 37°C were about 25% of the values measured at room temperature, consistent with the inability of p2Ca to stabilize L^d at 37°C (ref. 23). Attachment was minimal at 4°C, even after prolonged incubation. CD8 requirement for cell capture was tested by measuring attachment of purified CD8$^+$ 2C T cells. p2Ca and dEV-8 required CD8, whereas QL9 and SIYR did not. Thus, CD8 requirement was not completely correlated with TCR-ligand affinity.

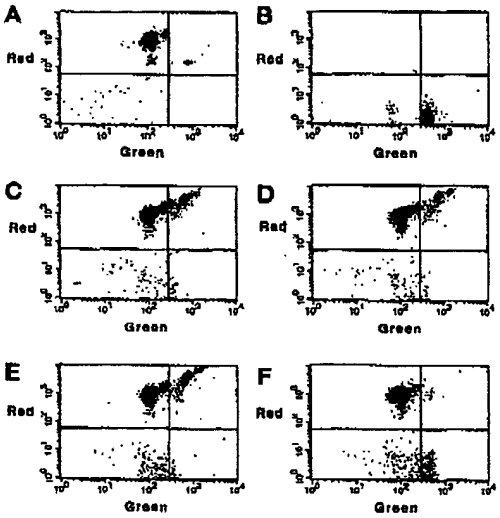

Figure 1. Complex formation between 2C T cells and L^d-coated beads using green versus red fluorescence dot plots. (A) Beads alone. (B) Cells alone. (C) Cells, beads, and QL9 peptide. (D) Cells, beads, and p2Ca peptide. (E) Cells, beads, and SL9 peptide. (F) Cells, beads, and LCMV peptide.

Recovery of 2C T cells mixed with irrelevant CD8$^+$ T cells. To assess whether MHC-coated beads could be used for T-cell precursor enrichment, we mixed fluorescein-labeled 2C T cells with CD8$^+$ T cells from naive C57BL/6 mice. After incubation with MHC-coated beads and peptide, adsorbed cells were eluted and counted, and the percentage of 2C T cells was determined by flow cytometry (Fig. 4). While 2C T cells were undetectable at the initial frequency of 0.03%, a definite peak of green fluorescence, representing 65.1% of the recovered cells, was observed following adsorption. This peak displayed the same fluorescence intensity as the original fluorescein-stained 2C T-cell population. No peak was recovered when a control peptide was used. A 800- to 1600-fold enrichment in 2C T cells in comparable experiments using beads coated with three different MHC-peptide complexes was attained (Table 2). Cell recovery was about 50% with peptide-MHC complexes of low affinity for the 2C TCR such as K^{bm3}-dEV-8 and K^b-SIYR, and reached 90–100% with the high-affinity L^d-QL9 complex. Final 2C T-cell purity was 47.6±2.1% when using K^b, the syngeneic restric-

Table 1. Quantitation of attachment of 2C T cells on L^d-coated beads loaded with various peptides.

Peptide	% cell captured (microscopy)	% cell captured (green/red fluorescence)	% cell captured (forward/side scatter)	2C TCR affinity for L^d peptide complex (M^{-1}) (refs. 8,9)	Peptide affinity for L^d (M^{-1}) (refs. 8,9,35)
QL9	87%	90.0%	81.0%	10^7	2×10^8
p2Ca	83%	85.7%	72.0%	2×10^6	4×10^8
SL9	77%	87.5%	N.D.	1.4×10^6	4×10^7
LCMV	<1%	2.2%	2.6%	$<10^5$	8×10^6
MCMV	<1%	N.D.	N.D.	$<10^5$	2×10^7

N.D.: not determined.

Table 2. Recovery of 2C T cells mixed with irrelevant CD8$^+$ T cells by using MHC-coated beads.

MHC molecule	Peptide	2C TCR affinity for MHC-peptide complex (M^{-1}) (ref. 9 and L.Teyton, unpublished data)	% 2C T cell before enrichment	% 2C T cell after enrichment	2C T cell enrichment	2C T cell recovery	Number of experiments
L^d	QL9	10^7	0.03%	24.8±6.9 %	828±230-fold	90.0±14.0%	3
K^{bm3}	dEV-8	1.8×10^6	0.03%	50.9±14.2 %	1697±473-fold	47.7±1.7%	2
K^b	SIYR	3.1×10^7	0.03%	47.6±2.1 %	1588±71-fold	56.8±0.6 %	2

tion element for the 2C TCR, and 24.8 ± 6.9% when using L^d, an allogeneic restriction element. This suggested that this difference could be accounted for by anti-L^d allogeneic T cells captured using L^d-coated beads. This would imply that some of the non-2C cells eluted from the beads were captured specifically. In all cases, the nonfluorescent cells in the eluted population represented only a minor fraction of the initial cell population (approximately 0.2%). In these experiments, 10^4 beads were incubated with 10^6 T cells, because this ratio was found to provide us with optimal cell recovery (data not shown).

Purification of antigen-specific T cells from naive mice. To investigate whether this method would be suitable to purify low-frequency T-cell precursors from naive animals, we incubated $CD8^+$ T cells from naive C57BL/6 mice with K^b-coated beads in the presence of either OVA-8 peptide or VSV-8 peptide. Following adsorption and in vitro expansion plus restimulation, we derived, respectively,

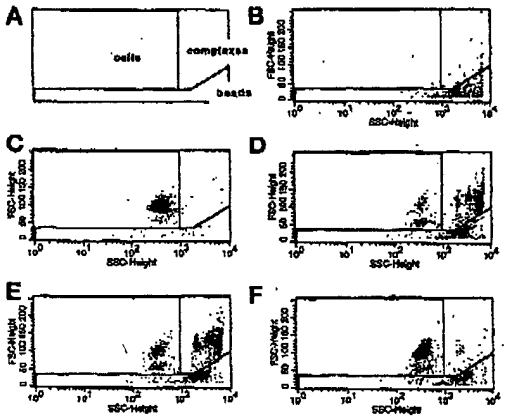

Figure 2. Complex formation between 2C T cells and L^d-coated beads using side scatter (SSC) versus forward scatter (FSC) dot plots. (A) Boundaries defining the three populations of events. (B) Beads alone. (C) Cells alone. (D) Cells, beads, and QL9 peptide. (E) Cells, beads, and p2Ca peptide. (F) Cells, beads, and LCMV peptide.

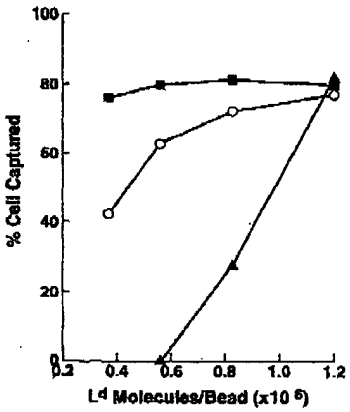

Figure 3. Complex formation between 2C T cells and beads coated with various amounts of L^d. 2C T cells were incubated with L^d-coated beads in the presence of QL9 peptide (■), p2Ca peptide (○), or SL9 peptide (▲).

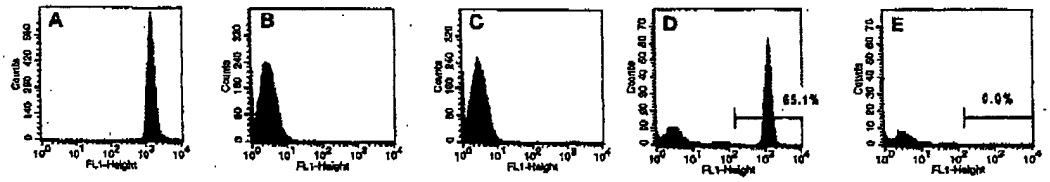

Figure 4. Enrichment of antigen-specific T cells using capture on MHC-coated beads. Green fluorescence (FL1) histograms of cell populations before and after enrichment. (A) Fluorescein-labeled 2C T cells. (B) Unlabeled purified $CD8^+$ T cells from naive C57BL/6 mice. (C) Mixture of 99.7% unlabeled $CD8^+$ T cells and 0.03% fluorescein-labeled 2C T cells. (D) Cells recovered after adsorption on K^{bm8}-coated beads in the presence of dEV-8. (E) Cells recovered after adsorption on K^{bm8}-coated beads in the presence of E1.

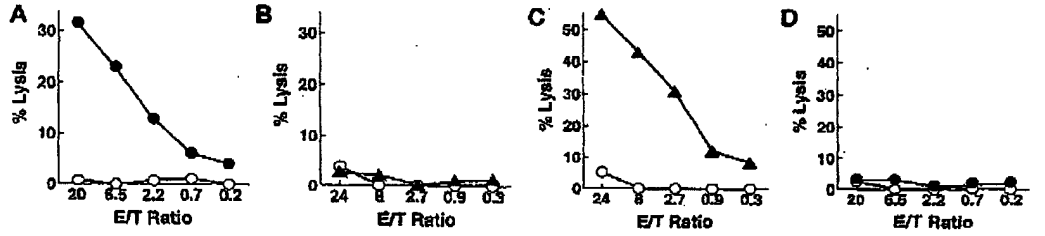

Figure 5. Expansion of specific CTL from naive mice after enrichment using MHC-coated beads $CD8^+$ T cells from C57BL/6 mice were adsorbed onto K^b-coated beads in the presence of (A and B) OVA-8 or (C and D) VSV-8. CTL were then derived by culture in K^b- and anti-CD28-coated plates in the presence of (A and D) 10 μM OVA-8 or (B and C) VSV-8. Cytotoxic activity was tested by using peptides (1 μM) as indicated: OVA-8: ●, VSV-8: ▲, no peptide: ○. Targets were EL4 cells ($H\text{-}2^b$). E/T = Effector/Target cell ratio.

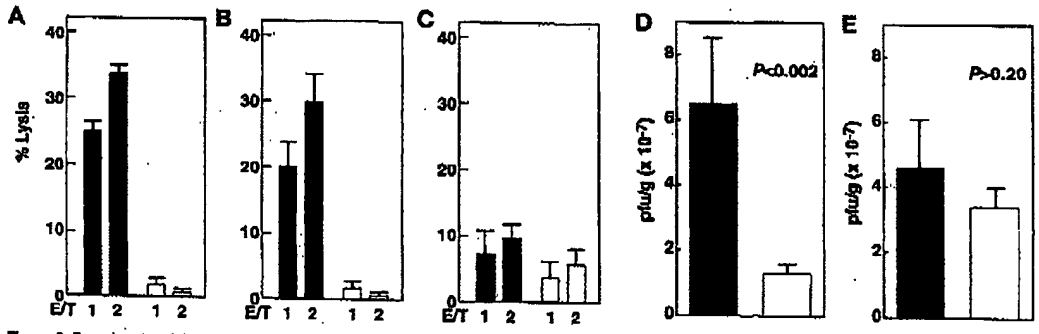

Figure 6. Functional activity of CTL derived from naive mice after enrichment using MHC-coated beads. In vitro and in vivo activity of CTL derived from CD8⁺ T cells from BALB/c mice after adsorption onto L^d-coated beads in the presence of LCMV peptide. (A) In vitro activity. Target cells: L^d-expressing RMA.S cells[25] pulsed with peptide (■: LCMV peptide; □: MCMV peptide). (B) In vitro activity. Target cells: BALB/c CL-7 ($H\text{-}2^d$) infected with LCMV Armstrong (■) or uninfected (□). (C) In vitro activity. Target cells: MC57 ($H\text{-}2^b$) infected with LCMV Armstrong (■) or uninfected (□). (D) In vivo activity. BALB/c mice ($H\text{-}2^d$); ■: no CTL injected; □: CTL injected. (E) In vivo activity. C57/BL6 mice ($H\text{-}2^b$); ■: no CTL injected; □: CTL injected.

OVA-8–specific CTL and VSV-8–specific CTL (Fig. 5A and C). Enrichment was specific as no VSV-8–specific CTL could be derived from cells captured using K^b–OVA-8–coated beads (Fig. 5B), and no OVA-8-specific CTL could be derived from cells captured using K^b-VSV-8-coated beads (Fig. 5D). We also derived anti-LCMV specific CTL after incubating $CD8^+$ T cells from BALB/c mice with L^d-coated beads in the presence of LCMV peptide. Such CTL specifically lysed target cells in the presence of LCMV peptide (Fig. 6A). We determined that for two independently generated CTL lines, avidities (defined as peptide concentrations required to reach half-maximal lysis) were, respectively, 2.5×10^{-11} M and 1.6×10^{-11} M. These were comparable to the avidities of previously described anti-LCMV CTL clones[34] that were in the 3×10^{-11} to 1×10^{-11} M range. Anti-LCMV CTL derived by magnetic capture also lysed virus-infected MHC-matched ($H\text{-}2^d$) fibroblasts in vitro (Fig. 6B), but not fibroblasts expressing another MHC haplotype ($H\text{-}2^b$) (Fig. 6C). Such CTL significantly ($p<0.002$) reduced virus titers in BALB/c mice ($H\text{-}2^d$) acutely infected with LCMV (Fig. 6D). This reduction was MHC-specific—no significant viral titer reduction ($p>0.20$) was observed in C57BL/6 mice ($H\text{-}2^b$) following CTL transfer (Fig. 6E).

To obtain an estimate of the CTLp frequencies after enrichment, we repeated the enrichment experiment using K^b-coated beads and OVA-8 peptide. The 12,000 cells recovered by adsorption onto K^b–OVA-8–coated magnetic beads were aliquoted and expanded separately in 12 wells of 96-well plates immediately after capture. Specific CTL were recovered in three wells, indicating that the precursor frequency after capture was approximately 1/3500. Similar results were obtained in three independent experiments. Thus, enrichment certainly had occurred as the precursor frequency for CTL anti–OVA-8 in naive C57BL/6 mice is 1/30,000 (ref. 25). However, these measurements in naive mice seem to indicate a smaller enrichment than that measured using 2C T cells mixed with irrelevant T cells (Table 2). This is not surprising because measurements with 2C T cells reflect directly the capability of the enrichment step, whereas, in naive mice, enrichment was likely underestimated. Some T cells might have been captured and expanded without displaying a cytotoxic activity, or might have been captured but would not grow in culture, or might have a too weak interaction with MHC-coated beads to be "capturable". It is unlikely that capture makes T cells unresponsive because 2C T cells can be expanded into CTL after capture.

Magnetic separation has proven to be the method of choice to purify rare cell populations[26–28], including peripheral blood hemopoietic progenitor cells (purification from 0.18%–54.4%, 300-fold enrichment, >39% recovery), peripheral blood burst-forming units-erythroid (purification from 0.04%–56.6%, 1400-fold enrichment, 13% recovery), and peripheral blood IgA,-expressing B lymphocytes (purification from 0.1–1.5% to up to 80%, up to 80% recovery). This magnetic separation procedure gives even better enrichment and recovery (Table 2). The purification methods mentioned above used antibodies as ligands for the specific cells; affinities of antibodies for antigens are several orders of magnitude higher than affinities of MHC-peptide complexes for TCR. Although fluorescent labeling of antigen-specific T cells is possible[29], cell sorting by flow cytometry could not be a substitute for magnetic separation. T-cell precursors are usually too rare to be detectable in flow cytometry, and the speed of analysis and sorting remains a limiting factor. In contrast, magnetic separation can be used to separate rare antigen-specific T cell populations, as well as to sort large numbers of cells quickly.

Experimental protocol

MHC production and immobilization. Soluble MHC molecules expressed in *D. melanogaster* cells[31] were purified[2] and biotinylated using biotin-BMCC (Pierce, Rockford, IL). Dynabeads M-500 (Dynal, Lake Success, NY) were covalently coupled to neutravidin (Pierce) via the p-toluene-sulphonyl chloride reactive groups present on their surface, exactly as specified by the bead manufacturer, using a ratio of 10 μg neutravidin/10^7 beads. Neutravidin-coated beads were then incubated with biotinylated MHC molecules for 2 h at 4°C under mild agitation. *Drosophila* cells express L^d and β_2-microglobulin (β_2m); the products are shown to be correctly folded heterodimers as they bind conformation-sensitive anti-L^d antibody 30-5-7 (ref. 30). In this article, L^d-β_2m heterodimers are referred to as L^d. Biotinylated L^d was used as a test model to study MHC molecule attachment to beads. Attachment of L^d increased with the amount of L^d between 0 and 1.5 μg of L^d/10^7 beads to reach a plateau at 3 μg of L^d/10^7 beads, as assessed by flow cytometry using anti-L^d antibody 30-5-7. This antibody is conformation sensitive[30]; therefore, we can infer that fully conformed class I heterodimer was conjugated to the beads. Nonbiotinylated molecules did not bind to beads. Saturation was attained with K^b and K^{mu} using the same range of concentrations as for L^d. $1.23 \pm 0.10 \times 10^8$ L^d molecules were immobilized per bead in saturating conditions, as determined by measuring the L^d concentrations in solution before and after binding using a solid phase immunoassay.

Peptides. The L^d- and K^b-binding peptides were synthesized on Applied Biosystem 430A and 431A instruments by standard solid-phase peptide synthesis method (tBoc chemistry). Peptide sequences were as follows: QL9: QLSPFPFDL; p2Ca: LSPFPFDL; SL9: SPFPFDLLL; p2Ca-A3: LSAFPFDL; dEV-8: EQYKFYSV; SIYR: SIYRYYGL; LCMV: RPQASGVYM; MCMV: YPHFMPTNL; OVA-8: SIINFEKL; VSV-8: RGYVYQGL; and E1: EIINFEKL.

Cell purification. Mice were kept under specific pathogen-free conditions. 2C transgenic mice were bred to the C57BL/6 strain, and progeny were selected for expression of the 2C TCR. Inguinal, axillary, cervical, iliac, and mesenteric lymph nodes were dissected and separated into single-cell suspension. Cells were purified at 4°C under sterile conditions by negative selection using Dynabeads coated with goat antimouse Ig (Southern Biotechnology, Birmingham, AL), anti-CD4 (H129.19; Gibco BRL, Gaithersburg, MD), or anti-MHC class II (for C57BL/6 and 2C transgenic mice: anti-I-A^b: AF6-120.1 and KH74, Pharmingen, San Diego, CA; for BALB/c mice: anti-I-A^d ; 34-5-3, Pharmingen). Purified cells from normal mice were 90–94% CD8$^+$. Purified cells from transgenic mice were 97–98% reactive with the anticlonotypic antibody 1B2 (*ref.* 31) (hybridoma from Dr. David Kranz, University of Illinois, Champaign, IL); 2C CD8$^+$ (CD4$^-$) T cells were prepared by depleting purified 2C T cells using the anti-CD8a antibody 53-6.7 (Gibco-BRL).

Cell capture on MHC-coated beads. Cells (10^6/ml in Fig. 1, Fig. 2, and Table 1; 10^5/ml otherwise) were incubated for 3 h with MHC-coated beads (10^7/ml) in the presence of 20 μM peptide at room temperature under mild agitation. To assess capture by microscopic examination (Table 1), cells adsorbed to beads were counted under the microscope. In cases where a definite rosette (3 beads or more per cell) was not observed, attachment was tested by gently tapping the coverslip. Capture was also assessed by flow cytometry (FACScan; Becton Dickinson, San Jose, CA) using two different approaches. In one approach, cell-bead complex formation was assessed using dot plots of green versus red fluorescence. 2C T cells were labeled in green with NHS-fluorescein (Pierce) and beads were labeled in red by coating with a mixture (9:1) of neutravidin (Pierce) and streptavidin-phycoerythrin (Pharmingen). Magnetic beads are autofluorescent; compensation was set so that phycoerythrin-stained beads displayed the same green fluorescence intensity as unstained beads. In the other approach, we used side scatter versus forward scatter dot plots, taking advantage of the fact that cells and beads form distinct populations of events under that representation.

Assessment of T-cell enrichment and recovery using T-cell capture on MHC-coated magnetic beads. 2C T cells were labeled with NHS-fluorescein and mixed with purified unlabeled CD8$^+$ T cells from naive C57BL/6 mice at a ratio of 1:3000; this mixture was incubated with MHC-coated beads in the presence of a peptide recognized by the 2C TCR or of a control peptide. Beads were then washed three times, and cells were eluted by overnight incubation at 4°C with 10 μg/ml of an anti-MHC antibody under mild agitation. Anti-MHC antibodies used were as follows: with L^d-coated beads, anti-L^d antibody 30-5-7 (ref: 30); with K^b and K^{bm1}-coated beads, anti-K^b antibody Y-3 (hybridoma from ATCC, Rockville, MD).

Expansion of cells captured from naive mice and tests for functional activity in vitro and in vivo. CD8$^+$ T cells were adsorbed on MHC-coated beads. Cell-bead complexes were recovered by washing the beads three times. CTL were then derived by culture in 96 well plates coated with the same MHC molecule and anti-CD28 antibody, in the presence of 10 μM peptide. At day 2, concanavalin A-activated supernatant was added (10% final); at day 9, cells were restimulated with spleen cells pulsed with 1 μM of the same peptide. At day 20, cytotoxic activity was tested by chromium release assay[32]. Targets were either cell lines pulsed with 1 μM peptide or infected with LCMV Armstrong (48 h; multiplicity of infection 1 pfu per cell). To test in vivo activity, cells were further expanded. Recipient mice were injected on day 0 with 2×10^5 pfu of LCMV Armstrong i.v.; on day 1, four LCMV-infected BALB/c mice were injected with 10^7 CTL anti-LCMV peptide, while four BALB/c mice received only PBS. As a control we used LCMV-infected C57BL/6 mice injected with either 10^7 CTL anti-LCMV peptide or PBS. On day 2, mice were sacrificed, and the spleens were assayed for infectious virus titers by plaque assay on Vero cells as reported[33]. Virus titers in the spleens were expressed as pfu per gram of tissue. Statistical analysis was performed using Student's *t* test[34].

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ANNEX 2

CTL assays with MLα018 cell line

E:T Ratio	Hodgkin's	ALL	Burkitt
10:1	86.2%	77.3%	60.8%
5:1	65.5%	55.7%	44%
2.5:1	40.4%	32.2%	23.1%
1:1	23.4%	16.6%	17.4%

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Evidence that HLA class II-restricted human CD4+ T cells specific to p53 self peptides respond to p53 proteins of both wild and mutant forms

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By stimulating peripheral blood mononuclear cells of four healthy donors with a mixture of overlapping peptides representing the core domain of p53, we established two CD4+αβ T cell clones and four lines that recognized wild-type and mutant p53 proteins as well as p53 self peptides in an HLA class II-restricted fashion. Two T cell lines established from two unrelated donors reacted to the p53 peptide (p)153–166 and p108–122, respectively, in the context of DP5 molecules. Two T cell clones established from two other unrelated donors were specific for p193–204 in the context of DRB1*1401 and for p153–165 in the context of DP5, respectively. These two T cell clones responded almost equally to both wild-type and four mutant recombinant p53 proteins. The proliferative responses of these T cell clones to p53 recombinant proteins were augmented by heat denaturing, thereby suggesting that altered conformation of the protein facilitates proteolytic processing to produce antigenic peptides. The DRB1*1401-restricted T cell clone specific for p193–204 killed a B lymphoblastoid cell line homozygous for HLA-DRB1*1401 when the cell line was pre-pulsed with p53 protein as well as peptide. These results indicate that CD4+ T cells reactive to p53 do exist in healthy individuals and the epitopes are probably ignored by the immune system under physiological conditions. It is suggested that such epitopes stimulate T cells to induce anti-p53 antibody production in cancer patients as previously reported by others. The possible involvement of p53-reactive T cells in anti-tumor immunity is discussed.

Key words: T cell / p53 tumor suppressor gene / Antigen presentation / Tumor immunity / HLA

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1 Introduction

Human tumor-associated antigens recognized by T cells and the epitopes of such antigens have been identified and characterized. The tumor-associated antigens are classified into tumor-specific shared antigens, differentiation antigens, antigens specific for individual tumors and ubiquitous antigens [1]. In some cases, the epitopes represent mutated gene products, but most of the epitopes recognized by T cells with anti-tumor activity were non-mutated products of tumor cells [2–4]. Such non-mutated tumor-related epitopes may derive from proteins produced more abundantly by tumor cells than by normal cells, or be generated by alteration of the antigen processing and presenting machinery.

[I 16819]

Abbreviations: BLCL: B lymphoblastoid cell line GM-CSF: Granulocyte-macrophage colony-stimulating factor

The T cell response requires proteolysis of antigenic proteins into peptides, which are presented by MHC class I molecules to CD8+ T cells, or by MHC class II molecules to CD4+ T cells. It is reasonable to speculate that CD8+ T cells directly lyse the tumor cells on which relevant peptide-MHC class I complexes are expressed. Likewise, CD4+ T cells may also directly recognize and attack tumor cells [5–7]. Indeed, expression of HLA class II molecules has been noted for some types of cancer cells [8]. Mutated p53 protein molecules that are intracellularly accumulated in such cancer cells may be transported to an HLA class II pathway. Even if tumor cells do not express MHC class II molecules, APC which have engulfed tumor cells should be capable of priming CD4+ T cells by presenting antigenic peptides in the context of their MHC class II molecules. In such a case, CD4+ T cells exhibit anti-tumor activity by secreting cytokines to activate immunocompetent cells such as CD8+ T cells, macrophages, NK cells and B cells [9]. In the local milieu around tumors, production of cytokines such

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as IFN-γ induced by inflammation and tissue destruction may promote the expression of MHC class II and co-stimulatory molecules on the surface of tumor cells as well as APC. In such situations, the tumor-specific antigen and peptides might be highly expressed on the surface of the tumor cell or APC that engulfed shed fragments of the tumor [10–15]. It is thus likely that the activated CD4⁺ T cells which are infiltrating the tumor play an important role in stimulating anti-tumor immune network systems to induce tumor regression.

Mutation of the p53 tumor suppressor gene was observed in 50–60 % of human cancers [16]. Most mutant p53 proteins become resistant to degradation by proteasomes and accumulate in the cytosol or nuclei of tumor cells [17, 18]. Anti-p53 antibodies were detected in sera of patients carrying cancer with an accumulated p53 protein. The antibodies were reactive to both mutant and wild-type p53 [19–22]. Most of the antibodies belong to the IgG isotype, the production of which requires T cell help [23], thereby suggesting that CD4⁺ T cells reacting to p53 are activated in such patients. Thus, p53 seems to be a good candidate for an antigen which stimulates anti-tumor immunity by CD4⁺ T cells.

While CD8⁺ T cells reacting to antigens specific for human tumors have been studied, little is known of CD4⁺ T cells recognizing tumor-specific antigen except for melanoma-specific T cells [24]. We recently reported evidence for a human CD4⁺αβ T cell clone recognizing both wild-type and mutant forms of p21 Ras protein in the context of HLA-DRB1*0101 molecules [25]. In the present work, we selected human p53 as a candidate antigen for anti-cancer immunity and established and analyzed CD4⁺ T cell clones recognizing p53-derived epitopes.

2 Results

2.1 Establishment of T cell lines and clones specific to p53 self peptides

Four T cell lines recognizing the mixture of p53 peptides without cysteine residue(s) were generated from four unrelated healthy donors. These T cell lines proliferated in response to a mixture of wild-type p53-derived peptides presented by autologous PBMC (Fig. 1). Both OT-1 and YT-3 lines strongly responded to a p53 peptide p147–166, whereas HF-5 and FT-5 lines did so to p186–205 and p105–123, respectively. Bulk lines, YT-3 and FT-5, showed slight responses even in the absence of synthetic peptides, probably because they included T cells reacting to antigens other than the synthetic peptides, such as self- or non-self-antigens which existed in the culture. OT-1 showed a trace response to p155–172, indicating that this line includes a small number of T cells reacting to p155–172 (Fig. 1B). In later experiments, we used only long-term cultured T cell lines stimulated repeatedly with the dominant T cell epitopes, and these bulk T cell lines responded only to the dominant epitope,

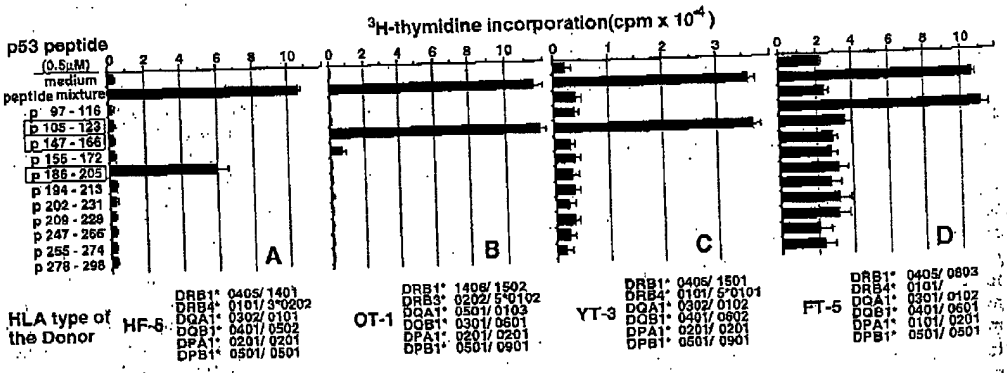

Figure 1. Identification of p53 peptide fragments carrying T cell epitopes for human CD4⁺ T cells in four unrelated healthy individuals. Proliferative responses of four independent T cell lines, (A) HF-5; (B) OT-1; (C) YT-3; and (D) FT-5, specific for a mixture of p53 peptides without cysteine residues, were investigated. To identify an individual peptide as a T cell epitope, each peptide was examined for its stimulating activity for these T cell lines. Peptides carrying T cell epitopes are boxed in the left column. T cell lines were cultured in the presence of each soluble peptide (0.5 μM) and irradiated autologous PBMC for 72 h. Values shown are the mean cpm of triplicate cultures ± SD. The HLA types of the T cell donors were determined at the DNA level and are given below each panel.

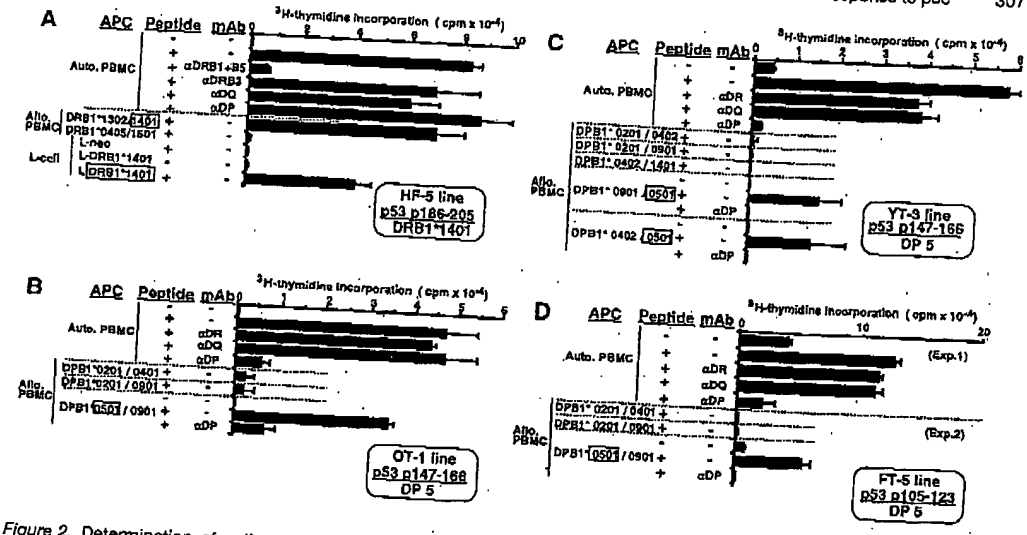

Figure 2. Determination of antigen-presenting HLA class II molecules in T cells specific for p53 self peptides. Proliferative responses of T cells to specific peptides were investigated in the presence of autologous PBMC and anti-HLA class II mAb, allogeneic PBMC or L-cells transfected with HLA genes. (A) T cell line HF-5, specific to p53 p186–205 peptide; (C) T cell line YT-3, specific to p147–166 peptide; and (D) T cell line FT-5, specific to p105–123 peptide. PBMC were pulsed with each peptide (0.5 μM) for 2 h followed by irradiation. To block the peptide presentation of APC, anti-HLA class II mAb were incubated with APC at 37 °C for 30 min before peptides were added. Boxed HLA alleles indicate antigen-presenting HLA class II molecules shared between T cell donors and APC donors. Mean cpm of duplicate responses ± SD is indicated. Panel (D) indicates two independent experiments done on different days.

without a significant background response. Subsequent limiting dilution of the HF-5 line gave rise to the T cell clone HF5.1 specific to p53 p186–205, and the OT-1 line gave rise to the T cell clone OT1.1 specific to p53 p147–166. Flow cytometric analyses of all T cell clones and lines revealed a $CD3^+$, $\alpha\beta TCR^+$, $CD4^+$, $CD8^-$ phenotype (data not shown).

2.2 Restriction molecules of p53-specific T cells

To identify the HLA molecule that presented the p53-derived peptides to established T cell lines and clones, blocking experiments using anti-HLA class II mAb were done. The response of T cell line HF-5 was completely blocked by HU-4 that recognizes DRB1 and DRB5 gene products [32, 33], but neither of HU-11 (specific for DQ1), HU-46 (specific for DQ4), B7/21 (specific for DP) and PLM-16 (specific for DRB3) were capable of blocking the response (Fig. 2A). Because the donor was typed for DRB1*0405, DRB4*0101, DRB1*1401 and DRB3*0202, the T cell clone was thought to be restricted by one of these two DRB1 molecules. As shown in Fig. 2A, allogeneic PBMC sharing the DRB1*1401 gene with the donor of HF-5 presented the peptides of HF-5, although allogeneic PBMC sharing the DRB1*0405 gene alone or carrying irrelevant DRB1 genes failed to do so. Furthermore, an L-cell transfectant expressing DRB1*1401 (L167-2) presented the peptides to HF-5, whereas an L-cell transfected with the neomycin-resistance gene alone (L-Neo) did not (Fig. 2A). These observations indicate that the T cell line HF-5 recognized the p53-derived peptide in the context of DRB1*1401. Similar data were obtained for the T cell clone HF5.1 (data not shown).

Using the same methods, restriction HLA class II molecules were identified in three other T cell lines as follows: Both T cell lines OT-1 and YT-3 which are heterozygous for DPB1*0501 and homozygous for the DPA1*0201 allele responded to p53 peptide p147–166 in the presence of allogeneic PBMC positive for DPA1*0201 and DPB1*0501, thereby indicating that these T cell lines were restricted by DP5 (DPA1*0201-DPB1*0501) (Fig. 2B, C). In Fig. 2D, allogeneic PBMC from the donor heterozygous for the DPB1*0501/0901 alleles and

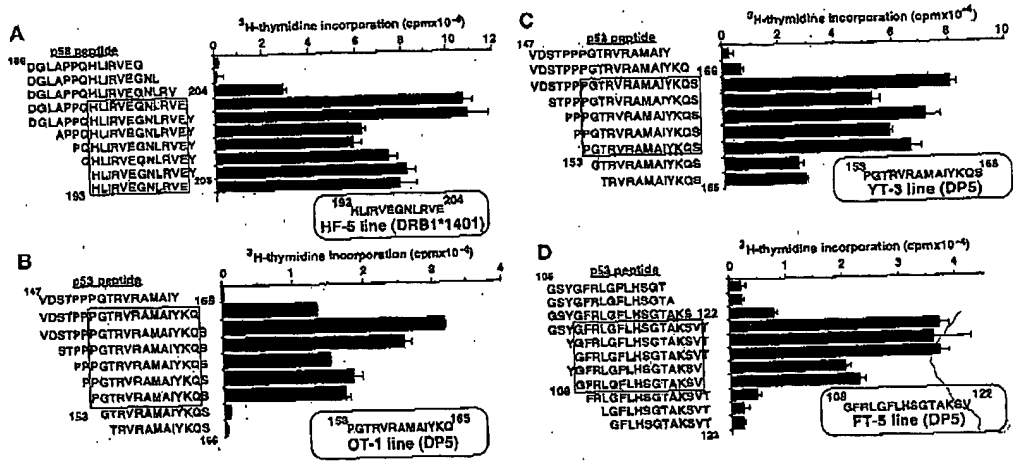

Figure 3. Identification of core peptide fragments for stimulation of four T cell lines, (A) HF-5; (B) OT-1; (C) YT-3; (D) FT-5. Various p53 peptides truncated either from the N terminus or from the C terminus of p53 self epitopes were investigated for their capacity to stimulate T cell lines. Autologous PBMC were used as APC, and T cells were cultured for 72 h in the presence of 0.5 μM of each truncated soluble peptide. Values shown are the mean cpm of triplicate cultures ± SD. Putative core sequences necessary to fully activate T cell lines are shown as boxed lettering, with restriction HLA class II molecules in each panel.

homozygous for DPA1*0201 allele presented the peptides to FT-5 established from the donor heterozygous for DPA1*0101/0201 and homozygous for DPB1*0501. Thus, FT-5 recognized p105–123 in the context of DP5 (DPA1*0201-DPB1*0501).

2.3 Identification of a core peptide fragment recognized by T cells

To identify a core sequence of the p53 peptide p186–205 (^{186}DGLAPPQHLIRVEGNLRVEY^{205}) for the DRB1*1401-restricted antigen presentation to HF-5 cells, a series of peptides truncated either from the C or N terminus were synthesized and reactivities of HF-5 were tested. As shown in Fig. 3A, deletion ^{204}E from the C-terminal end of the putative core sequence abrogated the proliferative response of HF-5. Moreover, an overlapping peptide p194–213, which does not habe ^{193}H at its N terminus, did not induce HF-5 to priferate (Fig. 1). These data indicate that the 12-mer p53 peptide p193–204 (^{193}HLIRVEGNLRVE^{204}) is a minimum sequence required for full activation of HF-5. By the same analyses, core sequences required for full activation of three other T cell lines proved to be: OT-1:p153–165 (^{153}PGTRVRAMAIYKQ^{165}) (Fig. 3B), YT-3:p153–166 (^{153}PGTRVRAMAIYKQS^{166}) (Fig. 3C), FT-5:p108–122 (^{108}GFRLGFLHSGTAKSV^{122}) (Fig. 3D). Although the same peptide mixture was used for the generation of T cell lines OT-1 and YT-3 and restriction HLA class II molecules are shared between the two lines, the C-terminal ^{166}S was required for recognition by YT-3 but not by OT-1.

2.4 Cytokine production by T cells in response to p53 peptides and proteins

As shown in Fig. 4, stimulation of the T cell clone HF5.1 with p53 p186–205 resulted in the production of a large amount of IFN-γ in the supernatants, whereas the production of IL-4 was much less. A similar pattern of cytokine secretion by T cell line YT-3 was observed in response to p53 p147–166. On the other hand, stimulation of the T cell clone OT1.1 with p53 p147–166 or T cell line FT-5 with p53 p105–123 resulted in the production of a large amount of IL-4 in the supernatants, whereas the production of IFN-γ was scanty. Furthermore, HF5.1 produced GM-CSF and TNF-α in recognition of p53 p186–205. A similar pattern of cytokine production was observed when HF5.1 was stimulated by p53 proteins (data not shown).

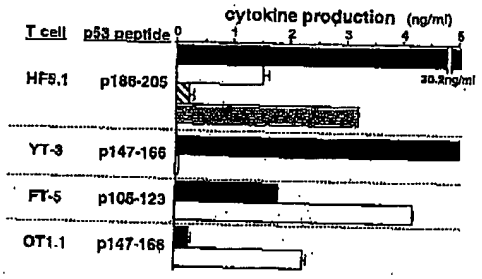

Figure 4. Cytokine production by HF5.1 and OT1.1 clones and YT-3 and FT-5 lines in response to specific p53 self peptides. T cell clones and lines were cultured with irradiated autologous PBMC in the presence of soluble peptides (0.5 μM each). After 24 h incubation, 100 μl from 200 μl of duplicate culture supernatants were collected for the measurement of IFN-γ (closed bar), IL-4 (open bar), TNF-α (hatched bar), and GM-CSF (gray bar) concentrations by ELISA, and mean concentrations are indicated. Results are expressed as the geometric means ± SD.

2.5 Proliferative responses of HF5.1 and OT1.1 clones to recombinant wild-type and various mutant p53 proteins

To determine if APC would process p53 protein into antigenic peptides to be recognized by established T cell clones, wild-type and mutant recombinant p53 proteins (V143A, R175H, R248W and R273H) were tested for their capacity to induce proliferative responses in HF5.1 and OT1.1. As demonstrated in Fig. 5A, wild-type p53 and four mutant p53 proteins but not irrelevant α4 protein stimulated proliferation of HF5.1 in a dose-dependent manner. There was no obvious difference in antigenicity among wild-type and the four mutant p53 proteins.

To confirm that the T cells recognize the peptide processed from proteins by APC but not peptides contaminating the protein preparations, we fixed APC with glutaraldehyde in various doses. Fixed APC could present peptides to stimulate T cell clone HF5.1, whereas they could not do so in the presence of protein (Fig. 5B), indicating that APC need intact antigen-processing activity to stimulate HF5.1 with p53 protein [42]. As shown in Fig. 6, another T cell clone, OT1.1, also responded to wild-type and all four mutant recombinant p53 proteins.

When recombinant proteins were boiled to alter their conformation, proliferative responses of HF5.1 and OT1.1 were augmented. Fig. 6B shows kinetics of proliferative responses of HF5.1 to p193–204, denatured p53 protein (R273H) and mutant native protein (R273H). Although the kinetics of responses differed between peptide antigen and protein antigens, there was no difference in kinetics between boiled and non-boiled proteins. The denatured protein reproducibly caused a larger proliferative response than the native protein.

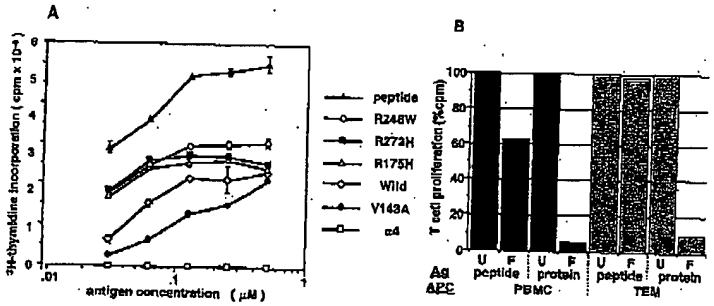

Figure 5. (A) Dose-dependent proliferative response of HF5.1 clone to wild-type or mutant recombinant p53 proteins V143A, R175H, R248W and R273H, respectively, an irrelevant α4 protein and p53 p186–205 peptide. Autologous PBMC (1.5×10^5) were used as APC, and HF5.1 (3×10^4) was cultured for 72 h in the presence of various concentrations of each recombinant protein or peptide. Values shown are the mean cpm of triplicate cultures ± SD. (B) Proliferative responses of HF5.1 to p53 p186–205 peptides or mutant proteins (R248W) presented by fixed or unfixed APC. Autologous PBMC and BLCL (TEM) were used as APC and were fixed with 0.0025 % and 0.01 % glutaraldehyde, respectively. The proliferative response of T cells induced by fixed APC is expressed as the percentage to that induced by unfixed APC. HF5.1 did not proliferate in the presence of fixed or unfixed APC without antigens. The 100 % cpm for each experiment were as follows: PBMC-peptide, 34 500 cpm; PBMC-protein, 10 100 cpm; TEM-peptide, 23 300 cpm; and TEM-protein, 5000 cpm.

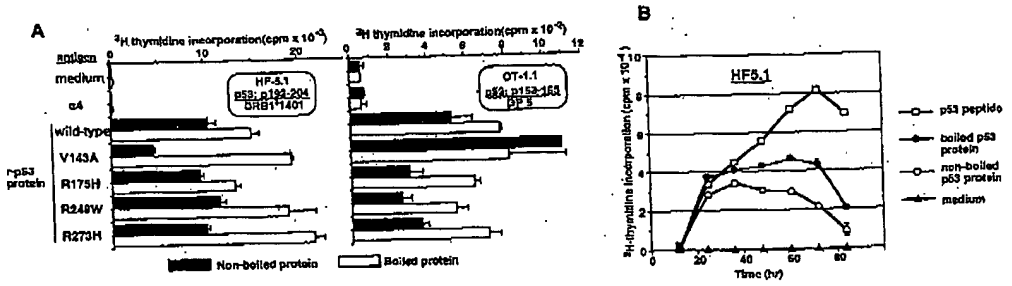

Figure 6. (A) Proliferative responses of HF5.1 and OT1.1 clones to boiled or non-boiled recombinant proteins. Recombinant proteins (either mutant p53, wild-type p53 or control α4) were boiled at 100 °C for 15 min and cooled on ice. After centrifugation at 13 000 × g for 5 min, supernatants were used for assay. Autologous PBMC were used as APC, and T cells were cultured for 72 h in the presence of 1.0 μM of each recombinant protein. Values shown are the mean cpm of triplicate cultures ± SD. (B) Kinetics of proliferative response of HF5.1 to p53 p193–204 peptide and boiled/non-boiled recombinant mutant p53 proteins (R273H). Cells were cultured with 0.5 μM peptide or 1.0 μM proteins and irradiated autologous PBMC for the indicated time period in the presence of 1 μCi/well of [^{3}H]thymidine during the final 12 h.

2.6 Cytotoxic activity of HF5.1

The cytotoxic activity of HF5.1 was then tested in a ^{51}Cr-release assay using BLCL pre-pulsed with p53 peptide or mutant protein (R248W) as a target. As shown in Fig. 7A, HF5.1 exerted cytotoxic potential on peptide-pulsed TEM, a BLCL bearing DRB1*1401, but not in KT 2 which is homozygous for an irrelevant DRB1*0406 allele. A similar cytotoxicity pattern was observed when the target cells were pulsed with p53 protein (Fig. 7B). When we did blocking experiments using either anti-Fas plus anti-Fas-ligand mAb or anti-TNF-α mAb to examine the mechanism of cytotoxic activity, these antibodies did not reproducibly block the cytotoxicity (data not shown).

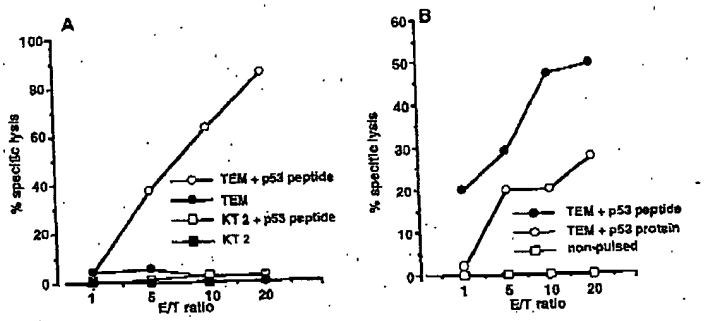

Figure 7. (A) Lytic activity of HF5.1 clone against DRB1*1401-positive BLCL, TEM, but not against DRB1*0406-homozygous KT2 pre-pulsed with p53 peptide p186–205. ^{51}Cr-labeled cells (2 × 10^3/well) were incubated with or without 10 μM p53 peptide at 37 °C for 2 h and washed with RPMI 1640 medium to be used as target cells. (B) Cytotoxicity of HF5.1 against TEM pre-pulsed with p53 protein. Before ^{51}Cr labeling, TEM cells were pulsed with 1.0 μM mutant p53 protein (R248W) at 37 °C for 12 h and washed with RPMI 1640 medium. The HF5.1 clone was co-incubated with target cells at the indicated E/T ratio and ^{51}Cr release was measured after 4–6 h incubation. Values shown were calculated based on the mean cpm of triplicate cultures.

3 Discussion

It has been reported that mutant p53 proteins elicit humoral and cellular immune responses [19, 23, 44–51]. Mutated p53 may generate epitopes which stimulate the immune system, either because the mutations fall inside the epitope sequences or because they modify proteolytic processing of the p53 protein [47]. Patients with cancer, bearing mutated and intracellularly accumulated p53 protein, produce anti-p53 antibodies reacting to both mutant and wild-type p53 and they also exhibit a T cell proliferative response *in vitro* to wild-type p53 protein [52]. We reasoned that p53 includes some epitopes to which the immune system does not acquire tolerance, even though p53 is a ubiquitously expressed protein. In the present study, we identified several p53-derived epitopes to which T cells from healthy individuals responded.

T cells are thought to acquire tolerance against dominant self peptides that are effectively processed and presented abundantly by MHC molecules, while they may not be tolerant to subdominant self peptides lacking an efficient presentation [53, 54]. The p53-derived T cell epitopes used in the current study are considered to be subdominant self peptides of this type to which human T cells do not completely become tolerant. However, in the presence of a large amount of exogeneously added p53 protein, regardless of being wild-type or mutant, human T cells did respond. In addition, the subdominance of these epitopes may also be due to a lower expression level of HLA-DP on HLA class II-positive cells, including APC because three of four T cell epitopes identified in this study were restricted by HLA-DP.

Wild-type p53 protein is rapidly degraded by the ubiquitin-proteasome system; hence it has a short life span. Therefore, this protein is seldom processed and transported to MHC class II pathway in APC. On the other hand, some mutant p53 proteins acquire resistance to proteolysis by the ubiquitin-proteasome system and accumulate in the cytoplasm or nuclei of tumor cells bearing a mutated p53 gene [17]. The p53-derived epitopes may be presented by APC which engulf such tumor cells, or if the tumor cells express MHC class II molecules, then the accumulated p53 proteins may be incorporated into endosomes by auto-phagocytosis and be transported to the MHC class II pathway.

In the present study, we used core domain-derived peptides to establish CD4+ T cell lines or clones responding to p53 protein. The p53 core domain has been reported to be resistant to proteolytic processing producing antigenic peptides due to clustering of hydrophobic amino acid residues in this region and to its folding conformation [55–59]. It is assumed that peptides derived from the core domain are less likely to induce a tolerance in T cells than those derived from other domains, and that subdominant self peptides may be more frequent in this region. Indeed, HF5.1 and OT1.1 responded to the p53 peptide better than to recombinant p53 proteins at the same molar concentrations, suggesting that APC could not fully process p53 protein into an antigenic peptide recognized by these T cell clones. T cell clones HF5.1 and OT1.1 showed higher proliferative responses to heat-denatured p53 protein than they did to untreated protein (Fig. 6). This observation suggests that conformational changes in the p53 protein facilitate the proteolytic processing in APC to effectively produce the epitopes. The possibility that aggregation of boiled protein leads to an efficient phagocytosis by APC and subsequent higher responses of T cells would not be ruled out.

Several types of cancer vaccines are being tested. Priming of T cells by immunization with self protein-based vaccines is usually unsuccessful [53], probably because the immune system is already tolerant to dominant self epitopes which are effectively produced from self protein. On the other hand, T cells recognizing subdominant self peptides may not be tolerized [53, 60]. It is therefore conceivable that peptides corresponding to subdominant self peptides may function better as an anti-tumor vaccine than would whole protein, dominant self peptide or intact tumor cells expressing tumor-specific antigens [61]. Furthermore, T cells such as the p53-specific CD4+ T cell clone with both cytotoxic activity and Th1-like properties shown in the current study may directly facilitate tumor regression.

In this regard, the information we provided on the p53-derived epitopes to which healthy individuals can respond may be useful for developing anti-tumor vaccines.

4 Materials and methods

4.1 Peptides

Twenty-five overlapping peptides with 17–20 residues spanning the p53 core domain were designed according to wild-type p53 [26, 27]. The peptides were synthesized using a solid-phase simultaneous multiple peptide synthesizer (PSSM-8, Shimadzu Corporation, Kyoto, Japan), based on the 9-fluorenylmethyloxycarbonyl (Fmoc) strategy, and purified by C18 reverse-phase HPLC. The peptides used for primary stimulation of PBMC listed in Table 1 were divided into two groups, based on the presence of cysteine residue(s).

4.2 Recombinant proteins

The p53 expression plasmids (pGEX-3X, Pharmacia Biotech), encoding the wild-type and four types of mutants, V143A (standing for a p53 mutant carrying a Val to Ala replacement at residue 143), R175H, R248W and R273H, were kindly provided by Dr. S. Ishii, Laboratory of Molecular Genetics, Tsukuba Life Science Center, RIKEN and Dr. T. Matozaki, the Second Department of Internal Medicine, Kobe University School of Medicine [28, 29]. The irrelevant α4 cDNA cloned into pGEX-3X was kindly provided by Dr. S. Inui, Kumamoto University School of Medicine [30]. Expression and purification of glutathione S-transferase (GST)-p53 fusion protein were done essentially as described [31]. Briefly, GST-p53 and GST-α4 fusion proteins were expressed in *Escherichia coli* (HB101) with the addition of isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.1 mM. The final 1000 ml of bacterial culture were centrifuged and bacteria were resuspended in 20 ml PBS containing 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin and then stored at −80 °C. The bacteria pellet was then lysed by the X-press (BIOX) at −25 °C, Triton X-100 (Sigma) was added (final 1 %) and the lysate was incubated at 4 °C for 30 min. The recombinant lysate was mixed with glutathione-conjugated agarose beads and incubated in cleavage buffer containing Factor Xa (100 μg/ml) at 23 °C for 6 min. After incubation, the protein was concentrated and dissolved in culture medium. Proteins were checked by SDS-PAGE before being used in T cell proliferation assays.

4.3 Generation of T cell lines and clones specific for p53-derived self peptides

PBMC (1.5×10^5/well) from four unrelated healthy donors were incubated each with 0.5 μM of p53 peptide mixtures with or without cysteine residue(s), respectively, in RPMI 1640 medium (Gibco Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10 % pooled, heat-inactivated normal plasma in 96-well flat-bottom culture plates (Falcon, Becton Dickinson, Lincoln Park, NJ). In the case of a mixture of p53 peptides with cysteine residue(s), PBMC were incubated with peptides in the presence of 50 μM 2-ME. After 7–9 days, irradiated (3000 cGy) autologous PBMC (1.5×10^5/well) pulsed for 6 h with p53 peptide mixture (5 μM each) were added to culture wells carrying T cell blasts and were maintained for another 7 days. Aliquots of growing cultures were combined as a bulk T cell line for each donor, and reactivity against the mixture of overlapping peptides was determined. Cloning was done in Terasaki plates (Nunc, Roskilde, Denmark) by limiting dilution at 0.33 cells per well in the presence of irradiated (3000 cGy) autologous PBMC (3×10^4/well) pulsed with p53 peptide mixture (5 μM each), 100 U/ml human rIL-2 in the same medium as described above.

The cell surface markers of T cells were analyzed using direct immunofluorescence staining with anti-CD3, anti-CD4, anti-CD8 and anti-TCRα/β mAb conjugated with either FITC or PE (Becton Dickinson, San José, CA) and flow cytometry by FACScan.

4.4 Antigen-specific proliferative responses of T cells

Antigen-specific proliferation of the T cell lines and clones was assayed by culturing the T cells (3×10^4/well) in 96-well flat-bottom culture plates in the presence of various concentrations of antigenic peptide or protein and 3000 cGy-irradiated autologous or allogeneic PBMC (1.5×10^5/well) in multiplicate cultures. Cells were cultured for 72 h and during the final 16 h of 1 μCi/well of [^{3}H]thymidine and the incorporated radioactivity was measured by liquid scintillation counting. The following recombinant proteins were tested for antigenicity: wild-type p53 protein, mutant p53 proteins V143A, R175H, R248W and R273H, and an irrelevant protein α4 as a negative control.

To determine restriction molecules for antigen presentation, the T cell lines and clones were cultured with irradiated autologous PBMC, with or without saturating amounts of either anti-HLA class II mAb HU-4 (anti-HLA-DRB1 + DRB5 monomorphic) [32, 33], HU-11 (anti-HLA-DQ4 + DQ5 + DQ6) [34], HU-46 (anti-HLA-DQ4) [35], PLM-16 (anti-HLA-DRB3) [36] or B7/21 (anti-HLA-DP monomorphic) [32]. Allogeneic PBMC or mouse L cells transfected with HLA class II genes were also used as APC. HLA class II (DR,DQ,DP) alleles of cells used were determined at DNA level as described [37–39]. The L167-2 cell line transfected with the DRA + DRB1*1401 genes [40] was a generous gift from Dr. H. Inoko, Tokai University, Isehara, Japan. All L cells, including those transfected with selection marker Neo^r gene alone (L-Neo) and used as a control, were treated with 20 μg/ml mitomycin C for 30 min before use, as described [41], followed by exposure to 10 μM peptides for 2 h.

In some experiments, APC were fixed glutaraldehyde as follows: APC were washed and resuspended in PBS at 10^7 cells/ml. Diluted glutaraldehyde (Sigma) was added to the cell suspension to the final concentration of 0.0025–0.001%, then incubated for 15 s at room temperature. An equal volume of 0.2 M L-lysine in PBS was then added, mixed gently, and incubated for 1 min at room temperature. APC were washed twice with PBS and used on the same day [42].

4.5 Quantitation of cytokines in T cell supernatants

Culture supernatants of the T cell clones and lines stimulated by APC plus antigens were collected after 24 h of stimulation and stored in aliquots at −80 °C until determinations of lymphokine concentrations. The granulocyte-macrophage colony-stimulating factor (GM-CSF) ELISA kit

Table 1. Amino acid sequences of synthetic overlapping peptides representing the core domain of wild-type p53 used for primary stimulation of PBMC

p53	p97-116	VPSQKTYQGSYGFRLGFLHS
p53	p105-123	GSYGFRLGFLHSGTAKSVT
p53	p113-132[a]	FLHSGTAKSVTCTYSPALNK
p53	p119-139a)	KSVTCTYSPALNKMFCQLAK
p53	p128-147[a]	PALNKMFCQLAKTCPVQLWV
p53	p137-150[a]	LAKTCPVQLWVDST
p53	p139-158[a]	KTCPVQLWVDSTPPPGTRVR
p53	p147-166	VDSTPPPGTRVRAMAIYKQS
p53	p155-174	TRVRAMAIYKQSQHMTEVVR
p53	p163-181[a]	YKQSQHMTEVVRRCPHHER
p53	p169-189[a]	TEVVRRCPHHERCSDSDGLA
p53	p178-197[a]	HERCSDSDGLAPPQHLIRV
p53	p186-205	GLAPPQHLIRVEGNLRVEY
p53	p194-213	LIRVEGNLRVEYLDDRNTFR
p53	p201-221	RVEYLDDRNTFRHSVVVPYE
p53	p209-228	NTFRHSVVVPYEPPEVGSD
p53	p217-236[a]	VVVPYEPPEVGSDCTTIHYNY
p53	p225-241[a]	VGSDCTTIHYNYMCNSS
p53	p229-249[a]	TTIHYNYMCNSSCMGGMNRR
p53	p239-258[a]	NSSCMGGMNRRPILTIITLE
p53	p247-266	NRRPILTIITLEDSSGNLLG
p53	p255-274	ITLEDSSGNLLGRNSFEVRV
p53	p263-282[a]	NLLGRNSFEVRVCACPGRDR
p53	p270-280[a]	EVRVCACPGRDRRTEEENLR
p53	p279-298	GRDRRTEEENLRKKGEPHHE

a) Since these peptides contained cysteine residues, PBMC were stimulated with the peptides in the presence of 50 μM 2-ME. The other peptides used did not contain cysteine residues and were co-cultured with PBMC in the absence of 2-ME.

TNF-α ELISA kit (ENDOGEN, Cambridge, MA), IFN-γ and IL-4 ELISA kits (Otsuka, Tokyo, Japan) were used according to the manufacturer's instructions.

4.6 Cytotoxicity assay

The cytotoxic activity of a CD4+ T cell clone was tested by ^{51}Cr-release assay. Cells of the EBV-transformed B lymphoblastoid cell line (BLCL) TEM, homozygous for HLA-DRB1*1401, and KT2, homozygous for HLA-DRB1*0405, were prepared as target cells. Target cells were labeled with ^{51}Cr and subsequently pulsed with 10 μM peptide. When the protein was used to pulse target cells, the cells were pre-incubated with 1 μM protein for 12 h before labeling with ^{51}Cr. Cells were plated in triplicate in 96-well round-bottom tissue culture plates and mixed with various numbers of effector cells. After 4–6 h of incubation, radioactivity in the supernatants of each well was measured. The spontaneous release from each target cell was usually less than 20–25 % of the maximum release (induced by treatment with 0.1 % Triton X-100). The result was calculated as: % specific lysis of the target cell = (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100.

To examine the mediator of cytotoxic activity, HF5.1 and TEM were pretreated with various concentrations of anti-Fas ligand mAb NOK2 [43], anti-Fas mAb ZB4 (MBL) and anti-TNF-α mAb B-C7 (Serotec). mAb NOK2 was kindly provided by Dr. H. Yagita, Department of Immunology, Juntendo University School of Medicine.

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ANNEX 3

Expression of hSIMP in cancer cells